PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 May 2000 (12.05.00)	
International application No. PCT/US99/17776	**Applicant's or agent's file reference** FP-68285/RMS
International filing date (day/month/year) 06 August 1999 (06.08.99)	**Priority date** (day/month/year) 06 August 1998 (06.08.98)
Applicant LUO, Ying et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 March 2000 (06.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Kiwa Mpay
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

WO 00/07545
PCT/US99/17776

PATENT COOPERATION TREATY

09/762491

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
SILVA, Robin, M.
Flehr Hohbach Test Albritton & Herbert LLP
Suite 3400
4 Embarcadero Center
San Francisco, CA 94111-4187
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 17 February 2000 (17.02.00)	
Applicant's or agent's file reference FP-68285/RMS/SSR	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/17776	06 August 1999 (06.08.99)	06 August 1998 (06.08.98)

Applicant
RIGEL PHARMACEUTICALS, INC. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   17 February 2000 (17.02.00) under No. WO 00/07545

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Col mbettes** **1211 G neva 20, Switz rland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/308 (July 1996)

3099983

INTERNATIONAL SEARCH REPORT

Inter. ..nal Application No
PCT/US 99/17776

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/12 C12N5/10 C07K16/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, SCISEARCH, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "Human cDNA clone IMAGE:667313 5'" EMBL SEQUENCE DATABASE, 27 February 1997 (1997-02-27), XP002141903 HEIDELBERG DE Accession Nr.: AA227673; 99,3% identity seq.id.no.5, nucl. 940-1380 abstract --- -/--	2,5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2000	20/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/17776

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "Human cDNA clone IMAGE:310581, 3'" EMBL SEQUENCE DATABASE, 19 April 1996 (1996-04-19), XP002141904 HEIDELBERG DE Accession No.: N99896 94,5% identity seq.id.no.5, nucl. 1289-1557 abstract	2,5
A	WO 96 36730 A (GEN HOSPITAL CORP) 21 November 1996 (1996-11-21) the whole document, especially page 13, lines 14-20 and page 20, line 17 - page 21, line 9	1,18
A	WO 97 15586 A (TULARIK INC) 1 May 1997 (1997-05-01) the whole document	1,18
A	COHEN G M: "Caspases: the executioners of apoptosis" BIOCHEMICAL JOURNAL,GB,PORTLAND PRESS, LONDON, vol. 326, 1997, pages 1-16, XP002107845 ISSN: 0264-6021 abstract; figure 4	1,17-19
P,X	STRAUSBERG R ET AL.: "Human cDNA clone IMAGE:2108297, 3'" EMBL SEQUENCE DATABASE, 5 February 1999 (1999-02-05), XP002141905 HEIDELBERG DE Accession No.: AI394293 100% identity seq.id.no.5, nucl. 1240-1557 abstract	2,5

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter...onal Application No
PCT/US 99/17776

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636730 A	21-11-1996	US 5674734 A	07-10-1997
		AU 707598 B	15-07-1999
		AU 5487396 A	29-11-1996
		CA 2219984 A	21-11-1996
		EP 0852627 A	15-07-1998
		JP 11506317 T	08-06-1999
WO 9715586 A	01-05-1997	AU 7457796 A	15-05-1997

PATENT COOPERATION TREATY

PCT

09/762491

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
FP-68285/RMS		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/17776	06/08/1999	06/08/1998
Applicant		
RIGEL PHARMACEUTICALS, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   APOPTOSIS PROTEINS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/17776

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/12 C12N5/10 C07K16/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, SCISEARCH, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2000	20/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	De Kok, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/17776

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "Human cDNA clone IMAGE:310581, 3'" EMBL SEQUENCE DATABASE, 19 April 1996 (1996-04-19), XP002141904 HEIDELBERG DE Accession No.: N99896 94,5% identity seq.id.no.5, nucl. 1289-1557 abstract	2,5
A	WO 96 36730 A (GEN HOSPITAL CORP) 21 November 1996 (1996-11-21) the whole document, especially page 13, lines 14-20 and page 20, line 17 - page 21, line 9	1,18
A	WO 97 15586 A (TULARIK INC) 1 May 1997 (1997-05-01) the whole document	1,18
A	COHEN G M: "Caspases: the executioners of apoptosis" BIOCHEMICAL JOURNAL,GB,PORTLAND PRESS, LONDON, vol. 326, 1997, pages 1-16, XP002107845 ISSN: 0264-6021 abstract; figure 4	1,17-19
P,X	STRAUSBERG R ET AL.: "Human cDNA clone IMAGE:2108297, 3'" EMBL SEQUENCE DATABASE, 5 February 1999 (1999-02-05), XP002141905 HEIDELBERG DE Accession No.: AI394293 100% identity seq.id.no.5, nucl. 1240-1557 abstract	2,5

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/17776

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636730 A	21-11-1996	US 5674734 A	07-10-1997
		AU 707598 B	15-07-1999
		AU 5487396 A	29-11-1996
		CA 2219984 A	21-11-1996
		EP 0852627 A	15-07-1998
		JP 11506317 T	08-06-1999
WO 9715586 A	01-05-1997	AU 7457796 A	15-05-1997

ID HS1147286 standard; RNA; EST; 440 BP.
XX
AC AA227673; XP-002141903
XX
SV AA227673.1
XX
DT 27-FEB-1997 (Rel. 51, Created)
DT 03-MAR-2000 (Rel. 62, Last updated, Version 3)
XX
DE zr55d09.r1 Soares_NhHMPu_S1 Homo sapiens cDNA clone IMAGE:667313
DE 5', mRNA sequence.
XX
KW EST.
XX
OS Homo sapiens (human)
OC Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Teleostomi;
OC Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
XX
RN [1]
RP 1-440
RA Hillier L., Allen M., Bowles L., Dubuque T., Geisel G., Jost S., Kucaba T.
RA Lacy M., Le N., Lennon G., Marra M., Martin J., Moore B., Schellenberg K.,
RA Steptoe M., Tan F., Theising B., White Y., Wylie T., Waterston R.,
RA Wilson R.;
RT "WashU-Merck EST Project 1997";
RL Unpublished.
XX
DR RZPD; IMAGp998B181628; IMAGp998B181628.
DR RZPD; IMAGp998B181628Q6; IMAGp998B181628Q6.
XX
CC On Sep 12, 1996 this sequence version replaced gi:1392988.
CC Contact: Wilson RK
CC Washington University School of Medicine
CC 4444 Forest Park Parkway, Box 8501, St. Louis, MO 63108
CC Tel: 314 286 1800
CC Fax: 314 286 1810
CC Email: est@watson.wustl.edu
CC This clone is available royalty-free through LLNL ; contact the
CC IMAGE Consortium (info@image.llnl.gov) for further information.
CC Insert Length: 829 Std Error: 0.00
CC Seq primer: -28m13 rev2 ET from Amersham.
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FH Key Location/Qualifiers
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FT /db_xref="ESTLIB:589"
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FT /db_xref="RZPD:IMAGp998B181628Q6"
FT /note="Organ: mixed (see below); Vector: pT7T3D-Pac
FT (Pharmacia) with a modified polylinker; Site_1: Not I;
FT Site_2: Eco RI; Equal amounts of plasmid DNA from three
FT normalized libraries (melanocyte 2NbHM, pregnant uterus
FT NbHPU, and fetal heart NbHH19W) were mixed, and ss circles
FT were made in vitro. Following HAP purification, this DNA
FT was used as tracer in a subtractive hybridization reaction
FT
FT The driver was PCR-amplified cDNAs from pools of 5,000
FT clones made from the same 3 libraries. The pools consisted

P.D 27/02/1997
P. 1/2
(2)

FT of I.M.A.G.E. clones 260232-265223, 340488-345479, and
FT 484488-489479."
FT /organism="Homo sapiens"
FT /tissue_type="Pooled human melanocyte, fetal heart, and
FT pregnant uterus"
FT /lab_host="DH10B"
FT /clone="IMAGE:667313"
FT /clone_lib="Soares_NhHMPu_S1"
XX
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Hs1147286 Length: 440 May 25, 19100 16:53 Type: N Check: 7358 ..

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  51  TTAGGAGAAC CATAGAAAAC CAGCACTCTC GTAATGATGT CATGGTTTCT

 101  GAGTGGCTAA ACAAACTGAA TCTAGAGGAG CCTCCCAGCT CTGTTCCTAA

 151  AAAATGCCCG AGCCTTACCA AGAGGAGCAG GGACAAGAGG AGCAGGTTCC

 201  ACAAGCCTGG ACAGCAGGCA CATCTTCAGA TTCGATGGCC CAACCTCCCC

 251  AGACTCCAGA GACCTCAACT TTCAGAAACC AGATGCCCAG CCCTACCTCA

 301  ACTGGAACAC CAAGTCCTGG ACCCCGAGGG AATCAGGGGG CTGAGAGACA

 351  AGGCATGAAC TGGTCCTGCA GGACTCCGGA GCCAAATCCA GTAACAGGGC

 401  GACCGCTCGT TAACATATAC AACTGCTCTG GGGTGCAAGT
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ID HS896328 standard; RNA; EST; 481 BP.

XX

AC N99896; XP-002141904

XX

SV N99896.1

XX

DT 19-APR-1996 (Rel. 47, Created)

DT 04-MAR-2000 (Rel. 63, Last updated, Version 2)

XX

DE zb87d11.s1 Soares_senescent_fibroblasts_NbHSF Homo sapiens cDNA
DE clone IMAGE:310581 3' similar to contains element LTR1 repetitive
DE element ;, mRNA sequence.

XX

KW EST.

XX

OS Homo sapiens (human)

OC Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Teleostomi;
OC Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

XX

RN [1]

RP 1-481

RA Hillier L., Clark N., Dubuque T., Elliston K., Hawkins M., Holman M.,
RA Hultman M., Kucaba T., Le M., Lennon G., Marra M., Parsons J., Rifkin L.,
RA Rohlfing T., Soares M., Tan F., Trevaskis E., Waterston R., Williamson A.,
RA Wohldmann P., Wilson R.;

RT "The WashU-Merck EST Project";

RL Unpublished.

XX

DR RZPD; IMAGp998B22699; IMAGp998B22699.

XX

CC On Apr 14, 1993 this sequence version replaced gi:692807.
CC Contact: Wilson RK
CC Washington University School of Medicine
CC 4444 Forest Park Parkway, Box 8501, St. Louis, MO 63108
CC Tel: 314 286 1800
CC Fax: 314 286 1810
CC Email: est@watson.wustl.edu
CC This clone is available royalty-free through LLNL ; contact the
CC IMAGE Consortium (info@image.llnl.gov) for further information.
CC Seq primer: ETPrimer
CC High quality sequence stop: 67.

XX

FH Key Location/Qualifiers

FH

FT source 1. .481
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FT /db_xref="ESTLIB:423"
FT /db_xref="RZPD:IMAGp998B22699"
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FT polylinker V_TYPE: phagemid; Site_1: Not I; Site_2: Eco RI

FT 1st strand cDNA was primed with a Not I - oligo(dT) primer
FT [5' TGTTACCAATCTGAAGTGGGAGCGGCCGCATTTTTTTTTTTTTTTTTTT 3']

FT double-stranded cDNA was size selected, ligated to Eco RI
FT adapters (Pharmacia), digested with Not I and cloned into
FT the Not I and Eco RI sites of a modified pT7T3 vector
FT (Pharmacia). Library went through one round of
FT normalization to a Cot = 5. Library constructed by Bento
FT Soares and M.Fatima Bonaldo."

P.D. 19/04/1996 (2)
P. 1/2

FT /organism="Homo sapiens"
FT /clone="IMAGE:310581"
FT /clone_lib="Soares_senescent_fibroblasts_NbHSF"
FT /tissue_type="senescent fibroblast"
FT /lab_host="DH10B (ampicillin resistant)"
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SQ Sequence 481 BP; 91 A; 113 C; 134 G; 134 T; 9 other;

Hs896328 Length: 481 May 25, 19100 17:33 Type: N Check: 4127 ..

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 151  TGCTTTATTT CCCGCTATGA TTATACCAAC CCTGTGGCCT GCTCCAGGCT
 201  TCAGGATCTT TAGGGCCTTC TTGCGAACCT ACTGGTGGGG GGTGCTGCAA
 251  NCCCTCCCCT TGCCCGAAGN GCCAAGCCCC ATGTGGGGAA GGCAGTTGTC
 301  TGTTGCATAG TCAAGTAGTT GTTGTCTCCA ATTGCACCCC AGAGCAGTTG
 351  TATATGTTAA CGAGCGGTCN CCTGTTANTG GATTGGCNCG GGGTCTGCAN
 401  GCCAGTTCAT GCCTTGTTNT CAGCCCCTGA TTCCCGGGTC AGGAATTGTG
 451  NTCATTGAGT AGGTGGATCT GGTNTGAAAT T
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XP-002141905

P.D. 5/02/1999
P. 1/2 (2)

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ID   AI394293   standard; RNA; EST; 462 BP.
XX
AC   AI394293;
XX
SV   AI394293.1
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DT   05-FEB-1999 (Rel. 58, Created)
DT   03-MAR-2000 (Rel. 63, Last updated, Version 3)
XX
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DE   mRNA sequence.
XX
KW   EST.
XX
OS   Homo sapiens (human)
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Teleostomi;
OC   Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
XX
RN   [1]
RP   1-462
RA   NCI-CGAP;
RT   "National Cancer Institute, Cancer Genome Anatomy Project (CGAP), Tumor
RT   Gene Index http://www.ncbi.nlm.nih.gov/ncicgap";
RL   Unpublished.
XX
DR   RZPD; IMAGp998K185199; IMAGp998K185199.
XX
CC   On Jul 19, 1995 this sequence version replaced gi:2863656.
CC   Contact: Robert Strausberg, Ph.D.
CC   Tel: (301) 496-1550
CC   Email: Robert_Strausberg@nih.gov
CC   Tissue Procurement: Ash Alizadeh, John Byrd, M.D., Mike Grever,
CC   M.D., Louis M. Staudt, M.D., Ph.D.
CC   cDNA Library Preparation: M. Bento Soares, Ph.D.
CC   cDNA Library Arrayed by: Greg Lennon, Ph.D.
CC   DNA Sequencing by: Washington University Genome Sequencing Center
CC   Clone distribution: NCI-CGAP clone distribution information can be
CC   found through the I.M.A.G.E. Consortium/LLNL at:
CC   www-bio.llnl.gov/bbrp/image/image.html
XX
CC   Insert Length: 960   Std Error: 0.00
CC   Seq primer: -40UP from Gibco
CC   High quality sequence stop: 394.
XX
FH   Key             Location/Qualifiers
FH
FT   source          1. .462
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FT                   /db_xref="ESTLIB:1317"
FT                   /db_xref="RZPD:IMAGp998K185199"
FT                   /note="Vector: pT7T3D-Pac (Pharmacia) with a modified
FT                   polylinker; Site_1: Not I; Site_2: Eco RI; 1st strand cDNA
FT                   was primed with a Not I - oligo(dT) primer [5'
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FT                   T 3']; double-stranded cDNA was ligated to Eco RI adaptor
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 401  CAGGACCAGT TCATGCCTTG TCTCTCAGCC CCCTGATTCC CTCGGGGTCC
 451  AGGACTTGGT GT
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REVIEW ARTICLE

Caspases: the executioners of apoptosis

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Apoptosis is a major form of cell death, characterized initially by a series of stereotypic morphological changes. In the nematode *Caenorhabditis elegans*, the gene *ced-3* encodes a protein required for developmental cell death. Since the recognition that CED-3 has sequence identity with the mammalian cysteine protease interleukin-1β-converting enzyme (ICE), a family of at least 10 related cysteine proteases has been identified. These proteins are characterized by almost absolute specificity for aspartic acid in the P_1 position. All the caspases (ICE-like proteases) contain a conserved QACXG (where X is R, Q or G) pentapeptide active-site motif. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide (prodomain) together with one large and one small subunit. The crystal structures of both caspase-1 and caspase-3 show that the active enzyme is a heterotetramer, containing two small and two large subunits. Activation of caspases during apoptosis results in the cleavage of critical cellular substrates, including poly(ADP-ribose) polymerase and lamins, so precipitating the dramatic morphological changes of apoptosis. Apoptosis induced by CD95 (Fas/APO-1) and tumour necrosis factor activates caspase-8 (MACH/FLICE/Mch5), which contains an N-terminus with FADD (Fas-associating protein with death domain)-like death effector domains, so providing a direct link between cell death receptors and the caspases. The importance of caspase prodomains in the regulation of apoptosis is further highlighted by the recognition of adapter molecules, such as RAIDD [receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain]/CRADD (caspase and RIP adapter with death domain), which binds to the prodomain of caspase-2 and recruits it to the signalling complex. Cells undergoing apoptosis following triggering of death receptors execute the death programme by activating a hierarchy of caspases, with caspase-8 and possibly caspase-10 being at or near the apex of this apoptotic cascade.

INTRODUCTION

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in chemical-induced cell death [1–3]. Inappropriate apoptosis is implicated in many human diseases, including neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer [4,5]. Apoptosis is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations [1,6]. Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by dramatic stereotypic morphological changes in cell structure [7], suggesting the presence in different cells of a common execution machinery [8]. Apoptosis is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum (frequently in a subplasmalemmal distribution), a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells, so preventing an inflammatory response [1] (Figure 1). The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA [9], recognized as a 'DNA ladder' on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of apoptosis. These DNA ladders are derived from large fragments of DNA of 30–50 and 200–300 kbp, which may in terms of higher-order chromatin structure represent loops and rosettes of DNA [10–12]. Internucleosomal cleavage of DNA now appears to be a relatively late event in the apoptotic process, which in some models may be dissociated from early critical steps [13,14]. Nevertheless, its measurement is simple and it is often used as a major criterion to determine whether a cell is apoptotic.

Whereas early studies concentrated on the role of nucleases in apoptosis, more recently a role has been proposed for a number of different proteases, including serine proteases, calpains and proteasomes (reviewed in [15–18]). Most attention has focused on the interleukin-1β (IL-1β)-converting enzyme (ICE)-like proteases, due partly to the enormous progress made by Horvitz and his colleagues in understanding programmed cell death in the nematode *Caenorhabditis elegans*. During normal development, 131 cells of the 1090 cells generated die by apoptosis [2]. Two genes, *ced-3* and *ced-4*, are vital for cell death in *C. elegans*, while the *ced-9* gene antagonizes their function and prevents cell death [19]. The CED-9 protein bears sequence similarity to mammalian Bcl-2, which acts to prevent cell death in mammals. No mammalian homologue of CED-4 has yet been identified. The CED-3 protein bears marked sequence similarity to, and identity with, mammalian ICE [20]. This seminal finding, together with the observation that overexpression of ICE induces apoptosis, suggests that ICE may play a key role in the induction of apoptosis [20,21]. Further evidence supporting a critical role for

Abbreviations used: Ac, acetyl; AMC, 7-amino-4-methylcoumarin; CPP32, 32 kDa cysteine protease; CRADD, caspase and RIP adapter with death domain; DED, death effector domain; DNA-PK, DNA-dependent protein kinase; DNA-PK$_{cs}$, catalytic subunit of DNA PK; FADD/MORT1, Fas associating protein with death domain; ICE, interleukin-1β-converting enzyme; ICH, *Ice* and *ced-3* homologue; ICH-1$_L$ and ICH-1$_S$, long and short isoforms respectively of ICH-1; IL-1β, interleukin-1β; PARP, poly(ADP-ribose) polymerase; RAIDD, RIP associated ICH-1/CED-3 homologous protein with a death domain; Rb, retinoblastoma protein; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR-1, TNF receptor; TRADD, TNFR-1 associated death domain protein; U1 70 kDa, 70 kDa protein component of the U1 small nuclear ribonucleoprotein; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone.

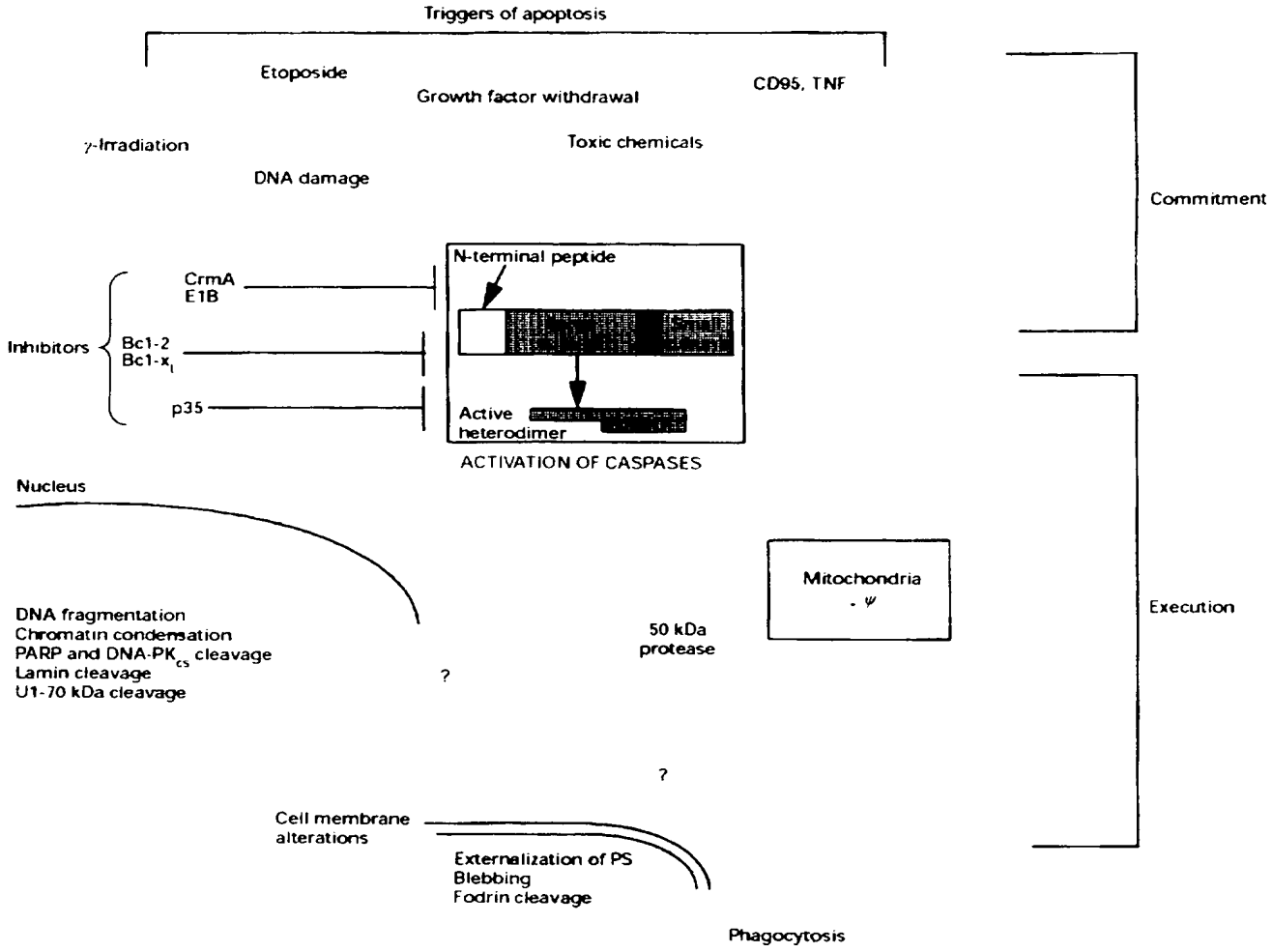


Figure 1 Activation of caspases represents a major control point in apoptosis

Apoptosis involves an initial commitment phase followed by an execution phase. Activation of the caspases results in nuclear, plasma-membrane and mitochondrial changes. The latter include the release of a 50 kDa protease, which may be responsible for some of the cellular changes associated with apoptosis. ψ, mitochondrial membrane potential; PS, phosphatidylserine. See the abbreviations footnote for other definitions.

Table 1 Members of the caspase family

Caspases-3, -7 and -9 have only one cleavage site between the large and small subunits, whereas the other caspases have two potential aspartate cleavage sites, resulting in removal of a linker region. Degrees of inhibition by cowpox viral serpin CrmA: + + +, potent inhibition; ±, very weak inhibition; ?, not known.

Caspase	Other names	Active site	Cleavage site(s) between large and small subunits	CrmA inhibition
Caspase-1	ICE	QACRG	WEHD ↓ S; FEDD ↓ A	+ + +
Caspase 2	Nedd2, ICH 1	QACRG	DQQD ↓ G; ELSD ↓ A	±
Caspase 3	CPP32, Yama, apopain	QACRG	IETD ↓ S	±
Caspase-4	ICE$_{rel}$II, TX, ICH 2	QACRG	WRVD ↓ S; LEED ↓ A	+ + +
Caspase-5	ICE$_{rel}$III, TY	QACRG	WRVD ↓ S; LEAD ↓ S	?
Caspase 6	Mch2	QACHG	DVVD ↓ N, TEVD ↓ A	±
Caspase 7	Mch3, ICE-LAP3, CMH 1	QACRG	IQAD ↓ S	±
Caspase 8	MACH, FLICE, Mch5	QACQG	VETD ↓ S, LEMD ↓ L	+ + +
Caspase-9	ICE-LAP6, Mch6	QACGG	DQLD ↓ A	?
Caspase 10	Mch4	QACQG	SQTD ↓ V; IEAD ↓ A	±

ICE-like proteases in apoptosis is the ability of specific protease inhibitors, including the cowpox viral serpin CrmA [21–23] and baculovirus p35 [24], to inhibit apoptosis (see below).

Since the recognition of the similarity between CED-3 and ICE in 1993, a further nine related ICE-like proteases have been identified. Several reviews have appeared on ICE and ICE-related proteases [7,15,16,25–29]. Due to the flurry of activity to isolate new family members, confusion has arisen as a result of different groups isolating the same protease. In order to resolve this, a unified nomenclature has recently been suggested [30] (Table 1). The trivial name proposed for all family members is caspase, the 'c' denoting a cysteine protease and the 'aspase' referring to the ability of these enzymes to cleave after an aspartic acid residue. Individual family members are then referred to in order of their publication, so ICE, the first family member, is caspase-1. Caspases are synthesized as inactive proenzymes, which are activated following cleavage at specific aspartate cleavage sites. Phylogenetic analysis of the caspases reveals that there are three subfamilies: an ICE subfamily, comprising

caspases-1, -4 and -5, a CED-3/CPP32 (32 kDa cysteine protease) subfamily, comprising caspases-3, -6, -7, -8, -9 and -10, and an ICH-1 (where ICH is *Ice* and *ced-3* homologue)/Nedd2 subfamily (Figure 2a and Table 2).

The presence of a family of structurally related caspases in cells raises a number of important questions in relation to their potential roles in cell death. (1) Are all caspases required for cell death, or are some members more important than others? (2) Do all modes of cell death utilize the same caspases? (3) Are caspases activated in series or in parallel? (4) Is one caspase at the apex of an apoptotic cascade? (5) How are these enzymes regulated in order to prevent their unwanted activation and the subsequent demise of the cell? (6) Are there normal cellular substrates for these enzymes? (7) What are their critical cellular substrates that lead to cell death? (8) Do specific caspases degrade specific proteolytic substrates during the apoptotic process? (9) Are the caspases pre-existing or are they synthesized in response to apoptotic stimuli? (10) Are the caspases tissue-specific? In this review, individual caspases will be discussed, paying particular attention to ICE, as most is known about this enzyme, and a detailed consideration of its properties is extremely helpful in understanding those of other family members. Then some of the known protein substrates of the caspases and their relationship with apoptosis will be reviewed.

ICE (CASPASE-1)

Structure and function

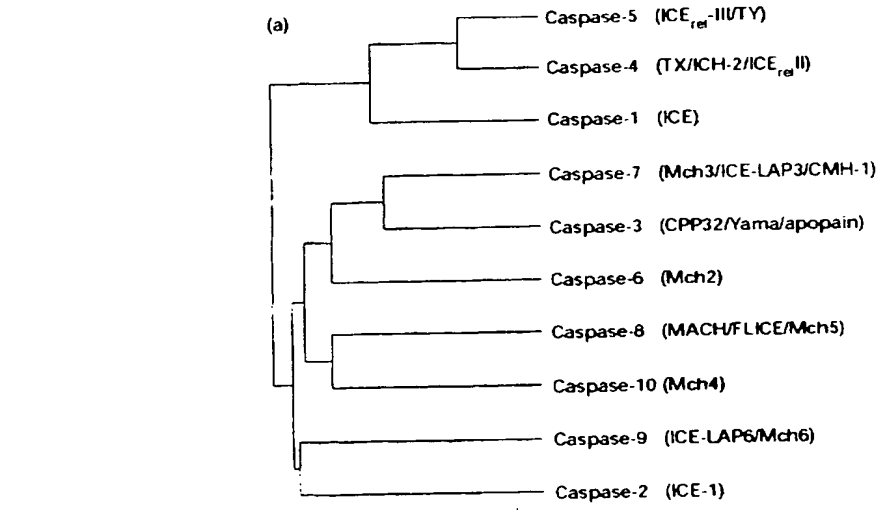
Early work on caspase-1 concentrated on its role in cleaving the inactive 31 kDa cytokine pro-IL-1β at Asp-116–Ala-117 to generate the active 17 kDa mature form of IL-1β, a key mediator of inflammation [31,32]. Purification and cloning of caspase-1 revealed that it is a 45 kDa protein (p45). Active ICE comprises two subunits of 20 kDa and 10 kDa (p20 and p10 respectively), both of which are required for catalytic activity and are derived from a single proenzyme following removal of an 11 kDa N-terminal peptide (prodomain) and a 2 kDa linker peptide (Figure 3) [33,34]. This scheme serves as a useful model for other caspases, although not all possess linker regions and the sizes of the prodomains vary (Figure 3). All four cleavage sites in p45, i.e. Asp-103, Asp-119, Asp-297 and Asp-316, arise at Asp–Xaa bonds (Figure 3), suggesting that active caspase-1 may be derived by autoproteolysis [33,34]. Following the initial cleavage at Asp-297–Ser-298, autoproteolysis occurs in a series of steps, generating fragments of increasing activity and eventually producing p20/p10 ICE [35,36]. Caspase-1 is found predominantly in the cytoplasm of cells as the p45 pro-form [37], although some is also localized to the external cell surface membrane, where it activates pro-IL-1β to its mature form during secretion [38]. Caspase-1 is a novel type of cysteine protease containing an active-site cysteine residue (Cys-285) in the p20 subunit, the mutation of which results in loss of activity. Substrate specificity studies revealed that caspase-1 has a strong preference for aspartic acid adjacent to the cleavage site in the P_1 position, a small hydrophobic amino acid (Gly or Ala) in the P'_1 position, and also a requirement for four amino acids to the left of the cleavage site [33,39]. Acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD.CHO, where CHO is aldehyde) and Ac-YVAD-7-amino-4-methylcoumarin (Ac-YVAD-AMC) were synthesized as a potent competitive reversible inhibitor and a fluorimetric substrate respectively for caspase-1 [33].

The X-ray crystal structure of caspase-1, in complex with specific tetrapeptide inhibitors that bind in the S_1–S_4 sites normally occupied by a peptide substrate, has been determined. The active enzyme is a tetramer of two p20 subunits surrounding two adjacent p10 subunits, with most of the area of contact between the dimers occurring between the p10 subunits [40,41]. Interactions between the p20 C-terminus and the p10 N-terminus also contribute to the stability of the homodimer. The active site spans both the p20 and p10 subunits, so explaining the requirement for both subunits for activity. Cys-285 and His-237 form a catalytic dyad in the active site of caspase-1. The active-site pentapeptide, Gln-Ala-Cys-Arg-Gly (QACRG), is in the p20 subunit. However, amino acid residues involved in forming the Asp pocket include Arg-179, Gln-283, Arg-341 and Ser-347, with only the first two residues being contributed by the p20 subunit. The two arginine residues (Arg-179 and Arg-341) form hydrogen bonds with the P_1 Asp residue of the substrate, and mutation of these residues results in the loss of catalytic activity [41]. Side chains of residues of p10 from Val-338 to Pro-343 interact with P_2–P_4 sites of the inhibitor [41]. Based on the crystal structure, two models were proposed for maturation of the proenzyme. In the first, two precursor p45 proteins associate and are then processed, with the p10 subunit from one caspase-1 molecule complexing with the p20 subunit from another caspase-1 molecule, so creating the active site. The alternative, but less favoured, model suggested processing followed by association of the subunits [41]. Using various active-site mutants, it has been shown that oligomerization of caspase-1 is required for autoprocessing [42] and that hetero-oligomerization may occur between different caspase homologues, but the *in vivo* relevance of this is not known. Using the yeast two-hybrid system, the prodomain of caspase-1 is absolutely required for dimerization and autoproteolysis, suggesting a regulatory rather than a structural role for the prodomain [43]. Using reverse transcriptase–PCR, four alternatively spliced isoforms of caspase-1 were identified that have differing effects on apoptosis [44].

Caspase-1 and apoptosis

Early studies pointed to a role for caspase-1 in the induction of apoptosis. CED-3 and caspase-1 share 28% sequence identity, and the active-site pentapeptide, QACRG, is completely conserved (Table 2) [20]. Overexpression of the murine ICE gene induces apoptosis in Rat-1 fibroblasts, which is abrogated by point mutations in the cysteine or glycine residues of the active-site pentapeptide and by either *bcl-2* or *crmA* [21]. The *crmA* gene (a cytokine response modifier gene) encodes a 38 kDa serpin that is a specific inhibitor of caspase-1, so preventing the proteolytic processing of IL-1β and thereby helping to suppress the response to viral infection [45]. As apoptosis is a major mechanism by which a host attempts to clear virally infected cells, viruses have evolved proteins that inactivate apoptosis in order to infect the host. Dorsal root ganglion neurons, which undergo apoptosis on withdrawal of nerve growth factor, are also protected by *crmA*, suggesting that caspase-1 may be involved in neuronal death in vertebrates [22].

Mice deficient in caspase-1 develop normally, appear healthy and are fertile, with no apparent abnormalities, suggesting that there are no gross defects in normal physiological processes involving apoptosis [46,47]. Macrophages from caspase-1$^{-/-}$ mice are equally susceptible to ATP-induced apoptosis as those from wild-type mice. Thymocytes from caspase-1$^{-/-}$ mice and wild-type mice show a similar susceptibility to apoptosis induced by dexamethasone or γ-irradiation, but thymocytes from the caspase-1$^{-/-}$ mice are somewhat more resistant to apoptosis induced by anti-CD95 (Fas/APO-1) antibody [46,47]. These results suggest that caspase-1 in itself is not involved in most forms of apoptosis, or that another caspase may substitute in the



(b)

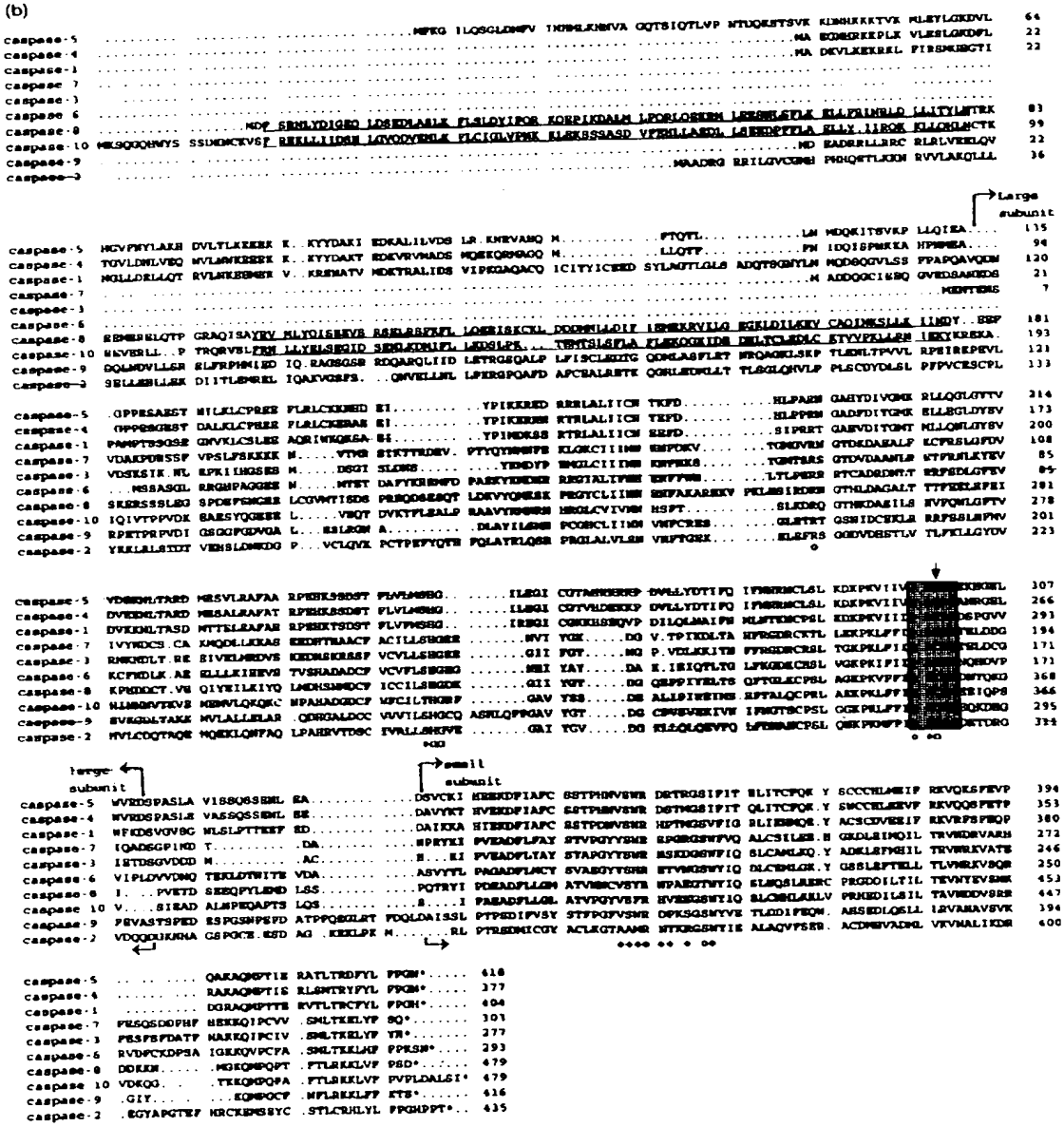


Table 2 Sequence identity of the caspases

The sequence identity between the full-length caspases shown in Figure 2(b) was analysed using the GAP program from the Genetics Compter Group.

	Identity (%)										
Caspase	1	2	3	4	5	6	7	8	9	10	CED 3
1	100	22	30	55	50	22	26	22	25	22	29
2		100	22	21	22	28	22	26	33	28	28
3			100	33	30	33	52	33	37	33	34
4				100	11	28	22	20	22	21	26
5					100	22	25	22	24	22	25
6						100	33	35	33	35	35
7							100	33	32	33	33
8								100	22	41	26
9									100	33	29
10										100	25

caspase-1 $^{-/-}$ mice. The only exception is the suggestion of a role for caspase-1 in CD95-induced apoptosis in thymocytes. However, no autoimmmune pathologies are seen in the caspase-1 $^{-/-}$ mice similar to those caused by the *lpr/lpr* mutation in the murine locus [48].

Further support for a role for caspase-1 in CD95-induced cell death was provided by the findings of a decrease in CD95-induced death due to CrmA, caspase-1 inhibitory peptides and caspase-1-specific antisense oligonucleotides [49,50], together with a small transient increase in a caspase-1-like activity prior to an increase in a CPP32 (caspase-3)-like proteolytic activity [51]. However, this elevation in a caspase-1-like activity has not been demonstrated to be essential for CD95-mediated apoptosis [51]. A role for caspase-1 has also been proposed in the apoptosis of mammary epithelial cells following loss of the extracellular matrix [52] and in DNA-damage-induced interferon regulatory factor-1-dependent T-lymphocyte apoptosis [53]. Similarly, caspase-1 is activated by growth factor deprivation, and suppression of this activation by growth factors such as insulin-like growth factor-1 and insulin also inhibits cell death [54]. In contrast, caspase-1 activity and apoptosis are uncoupled in macrophages undergoing apoptosis [55]. While there is some experimental support for a role for caspase-1 in apoptosis, in particular in CD95-mediated apoptosis, most data (see also below) suggest that other caspases may be of greater significance than caspase-1.

ICH-1/Nedd2 (CASPASE-2)

Nedd2 was originally identified using subtraction cloning as a developmentally down-regulated gene in mouse brain [56,57]. Using a murine *Nedd2* cDNA, a human foetal brain cDNA library was screened at low stringency, and *Ich-1*, the human homologue of *Nedd2*, was identified [58]. Both *Nedd2* and *Ich-1* encode proteins similar to caspase-1, and sequence alignment shows conservation of many important residues, including the active-site pentapeptide QACRG (Table 1 and Figure 2) [57,58]. The *Ich-1* mRNA is alternatively spliced into two forms, one encoding a protein of 435 amino acids (ICH-1_L) and the other encoding a protein of 312 amino acids (ICH-1_S; a truncated form of ICH-1_L) [58]. Overexpression of *Ich-*1_L in some, but not all, cell types results in apoptosis, whereas overexpression of *Ich-*1_S suppresses apoptosis induced by serum withdrawal, suggesting that *Ich-1* may play a role in both the positive and negative regulation of programmed cell death [58]. The enzymic activities of ICH-1_L/Nedd2 are required to cause cell death, as overexpression of mutant *Ich-*1_L*/Nedd2* (with a Ser or Gly respectively replacing the active-site Cys) results in loss of activity [57,58]. During embryonic development, *Nedd2* is expressed at relatively high levels in various tissues, including the central nervous system, liver, kidneys and lungs [57]. Both the kidney and central nervous system are associated with high levels of programmed cell death during development [59]. *Nedd2* is also expressed to varying extents in several adult tissues, including post-mitotic neurons [57].

Two recent studies have addressed the question of which Asp cleavage sites are important in the processing of Nedd2 [60,61]. The Asp-333 → Ala mutant lacks apoptotic activity and does not produce p20 and p10 fragments, suggesting it as the cleavage site at the C-terminus of p20. Asp-347 was identified as the Asp residue at the N-terminus of p10. Asp-135 appears to be a cleavage site upstream of p20, as cell death activity and processing are prevented when it is mutated [60,61]. The proposed cleavage sites in Nedd2 are conserved in caspase-2 (Figure 3). Caspase-2 may be activated *in vitro* by caspase-1, caspase-3 and granzyme B [61]. Recently, the cleavage of caspase-2 to its catalytically active subunits during the execution phase of apoptosis in the human monocytic tumour cell line THP.1 was demonstrated [62]. Caspase-2 was cleaved early during the apoptotic process, but it was not possible to discern whether its cleavage preceded that of other caspases. As yet, no specific intracellular protein substrates of caspase-2 have been identified.

CPP32/YAMA/APOPAIN (CASPASE-3)

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which are cleaved in many different systems during apoptosis (see below). Using the DNA sequence encoding the active site of caspase-1 and CED-3 to search an expressed sequence tag database, a human sequence was identified, cloned and shown to encode a 32 kDa cysteine protease, called CPP32 [63]. Independently, two other groups identified caspase-3, one naming it Yama (the Hindu god of death) and the other apopain [64,65]. Caspase-3, a member of the CED-3 subfamily of caspases (Table 2 and Figure 2b), is widely distributed, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system [63]. Based on the known cleavage site of PARP (DEVD↓G), Ac-DEVD-AMC was

Figure 2 (a) Phylogenetic relationships and (b) sequence alignment of the caspases

Phylogenetic relationships were determined and polypeptide sequences for the human forms of the caspases aligned using the PILEUP algorithm (Genetics Compuler Group, Madison, WI, U.S.A.). The relationships are based on the full length proenzymes, which is appropriate as the prodomains clearly play a functional role. The dendogram is not affected if the same analysis is carried out using caspases without their prodomains. Amino acid residues are numbered to the right of each sequence. The QACXG motif is conserved in all family members and is boxed. A coloured arrow above the aligned sequences indicates the position of the catalytic cysteine residue. Amino acids that align with residues within ICE that have been shown by the X-ray crystal structure to be involved in binding P_1 Asp (○), in catalysis (□) and adjacent to substrate P_2–P_4 amino acids (◆) are indicated. The underlined sequences represent the FADD/MORT1 (see the text) homology domains in caspase 8 and caspase 10.

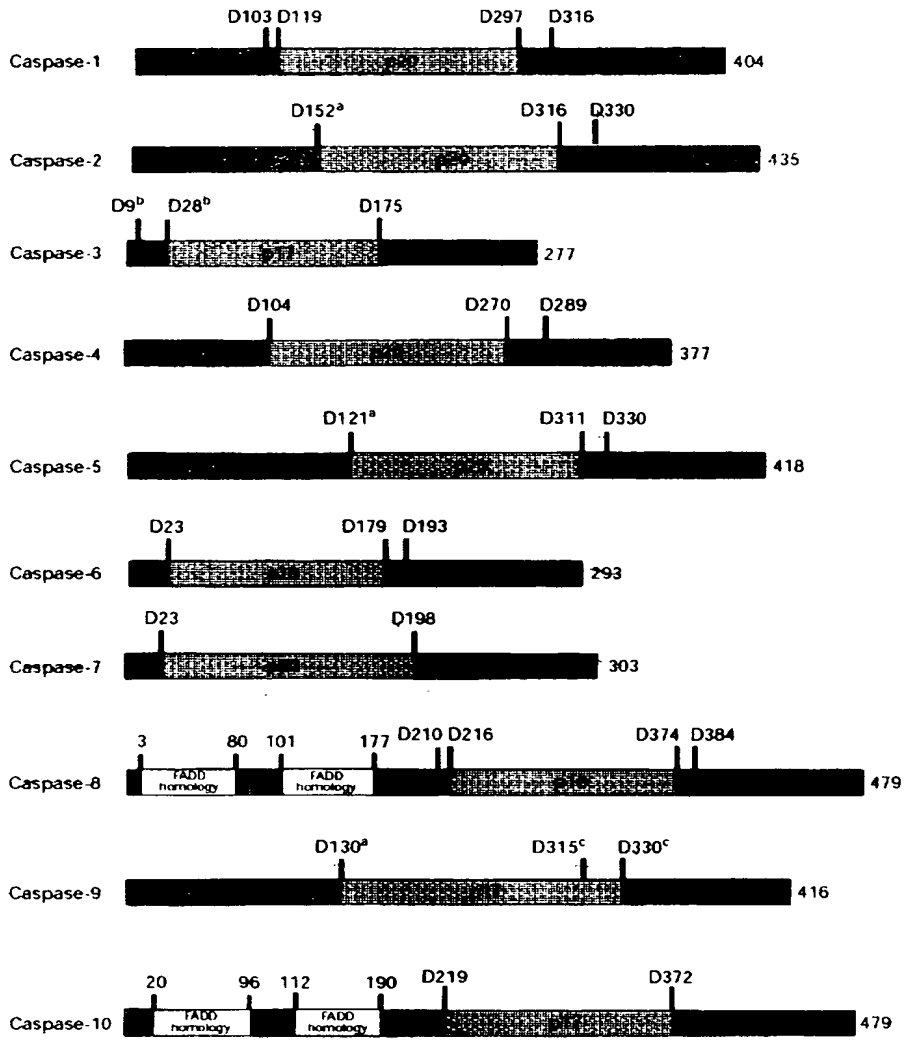


Figure 3 Proenzyme organization of the caspases

Caspases are synthesized as proenzymes, with a N terminal peptide or prodomain (PRO), and two subunits sometimes separated by a linker peptide (black box). Based on caspase 1 and caspase 3, active enzymes are heterotetramers of two large (~ 20 kDa; p20) and two small (~ 10 kDa; p10) subunits. The proenzymes are cleaved at specific Asp residues (D*n*, where *n* is the position in the protein). The numbers at the right-hand side are the numbers of amino acids in the protein. [a]Exact cleavage site not known; [b]the cleavage site of caspase-3 may be at Asp-9 or Asp-28 [65–67]; [c]caspase-9 is cleaved preferentially at Asp-330 by caspase 3 and at Asp-315 by granzyme B [82]. Caspase-2 cleavage sites are based on equivalent sites being present in Nedd2 [60,61]. †ADD represents the domains of caspase 8 and caspase 10 that are homologous to the DED of FADD/MORT1.

synthesized as a model substrate, and Ac-DEVD.CHO and its biotinylated derivatives were synthesized as specific inhibitors of PARP cleavage and as affinity ligands for purification of the protease. Using electrospray MS and N-terminal sequence analysis, the active enzyme was shown to be composed of two subunits of 17 kDa and 12 kDa, derived from the precursor protein by cleavage at Asp-28–Ser-29 and Asp-175–Ser-176 [65] (Figure 3). While the initial cleavage is probably between the large and small subunits, it has been suggested that processing within the prodomain occurs initially at Asp-9, not at Asp-28 [66,67].

During the execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contains a common Asp-Xaa-Xaa-Asp (DXXD) motif (Table 3), similar to that originally described in PARP [68]. In comparison with caspase-1, caspase-3 has no linker peptide and the prodomain is much shorter (Figures 2b and 3). Caspase-3 prefers a DXXD-like substrate, whereas caspase-1 prefers a YVAD-like substrate [65]. Both enzymes have an almost absolute requirement for an Asp in the P_1 position and both can tolerate a fair degree of substitution in the P_2 and P_3 positions, but in the P_4 position caspase-1 prefers a hydrophobic amino acid such as Tyr, whereas caspase-3 has a marked preference for an Asp. The three-dimensional structure of a complex of caspase-3 with DEVD.CHO, a potent tetrapeptide aldehyde inhibitor, shows that, although caspase-3 resembles caspase-1 in overall structure, its S_4 subsite is very different in size and chemical composition and accounts for their differences in specificity [69]. The S_4 subsite of caspase-1 is a large shallow hydrophobic depression that readily accommodates a tyrosyl side chain, while the analogous site in caspase-3 is a narrow pocket that closely surrounds the P_4 Asp side chain [69].

Table 3 Protein substrates of caspases

Abbreviation: SRLBP, sterol regulatory element binding protein.

Protein substrate	Cleavage motif	Caspase(s)	Function of substrate	References
PARP	DEVD ↓ G	3,7	DNA repair enzyme	68, 104
U1-70 kDa	DGPD ↓ G	3	Splicing of RNA	106
DNA PK_{cs}	DEVD ↓ N	3	DNA double-strand-break repair	106, 107
Gas2	SRVD ↓ G	?	Component of microfilament system	126
Protein kinase C δ	DMQD ↓ N	3	Cleaved to active form in apoptosis	130, 131
Pro-IL-1β	YVHD ↓ A	1	Cleaved to mature active cytokine	32–34
D4-GDP dissociation inhibitor	DELD ↓ S	3	Regulator of Rho GTPases	110
Lamin A	VEID ↓ N	6	Assist in maintaining nuclear shape	113–116
Heteroribonuclear proteins C1 and C2	?	3,7	Processing of pre mRNA	108
Huntingtin	DXXD	3	Huntington disease gene product	111
SRLBP 1 and SRLBP 2	DLPD ↓ S	3,7	Sterol regulatory element binding proteins	109
Fodrin	DETD ↓ S?	?	Membrane-associated cytoskeletal protein	127–129
Rb (see the text)	DLAD ↓ G	3	Cell cycle regulatory protein	112, 133, 134

The Trp residue at position 348 and an inserted 10-amino-acid sequence at position 381 (for ease of comparison, the residue numbers used are those of the analogous residues in caspase-1) play a crucial role in defining the size and shape of the S_4 subsite of caspase-3 and are also conserved in all known members of the CED-3 subfamily (Figure 2b). The activation of caspase-3 to either of its catalytically active p17 or p12 subunits has been demonstrated in cells undergoing apoptosis [70–72].

Caspase-3-deficient mice, generated by homologous recombination, are smaller than their littermates and die at 1–3 weeks of age. Thymocytes from caspase-3-deficient mice show a similar sensitivity to apoptosis induced by a number of different stimuli, including CD95, anti-CD3, staurosporine and dexamethasone. Brain development in these deficient mice is markedly affected, with a variety of hyperplasias being observed from embryonic day 12. Pyknotic clusters of apoptotic cells, observed at sites of major morphogenetic change in normal brain development, are not seen in the deficient mice, indicative of decreased apoptosis in the absence of caspase-3. This demonstrates that caspase-3 plays a critical role during morphogenetic cell death in the mammalian brain and also that mutation of a mammalian homologue of *Ced-3* leads to decreased cell death and a supernumerary cell population during development, emphasizing that the basic cell death machinery is evolutionarily conserved. The restricted phenotype also raises the possibility that other caspases may be important in other tissues or cell types [73].

Cytotoxic T lymphocytes kill target cells containing foreign antigens by either CD95- or granule-mediated cytotoxicity. Exocytosis of cytotoxic T lymphocyte granules allows perforin to polymerize in the target cell membrane, so facilitating entry of the granzymes, a family of serine proteases. Granzyme B also exhibits an unusual specificity for Asp in the P_1 position. *In vitro*, granzyme B can activate caspase-3, as assessed by formation of its p17 subunit as well as its ability to cleave PARP to its signature fragment [74,75]. Activation of caspase-3 by granzyme B is within the physiological range [75]. While granzyme B can activate directly caspase-3, it may also cleave another caspase which in turn activates caspase-3.

ICE_{rel}II/TX/ICH-2 (CASPASE-4) AND ICE_{rel}III/TY (CASPASE-5)

Three groups independently cloned ICE_{rel}II/TX/ICH-2 (caspase-4) [76–78] and two groups cloned ICE_{rel}III/TY (caspase-5) [76,79] (Figure 2b). Both caspase-4 and caspase-5 are members of the caspase-1 subfamily, and are more closely related to each other than to other homologues (Table 2). Caspase-4 expression, while lower than that of caspase-1, generally shows a similar tissue distribution, being found in most tissues examined with the exception of brain. Appreciable levels are found in both lung and liver, and also in ovary and placenta, where caspase-1 mRNA is barely detectable [76,77]. Caspase-5 is expressed at a much lower level than caspase-4 [76,79]. Caspase-4 and caspase-5 have different substrate specificities from that of caspase-1, being much poorer at cleaving pro-IL-1β [76–79]. Caspase-4 may be involved in the maturation of caspase-1 [78]. At high concentrations, caspase-4 cleaves PARP [80], but the biological relevance of this is unclear. Limited information is available concerning the precise sites at which caspase-4 and caspase-5 are processed. Maturation of caspase-1 results from cleavage after Asp residues at positions 119, 297 and 316, with the latter two residues being conserved in caspase-4 (Asp-270 and Asp-289) and caspase-5 (Asp-311 and Asp-330); by analogy, this probably represents the removal of a linker peptide (Table 1 and Figure 3). The Asp-119 site in caspase-1 is not conserved in caspase-4, but Asp-104 might define the cleavage site for removal of the prodomain [77]. Overexpression of caspase-4 and caspase-5 generally results in apoptosis, although in some studies apoptosis was only induced after removal of the prodomains, suggesting that the prodomains may be involved in the regulation of apoptosis [76,78].

Mch2 (CASPASE-6)

Using a PCR approach with degenerate primers encoding two highly conserved pentapeptides, QACRG and GSWFI, Alnemri and co-workers cloned Mch2 (a mammalian CED-3 homologue) from a human Jurkat T lymphocyte cDNA library [81]. Two transcripts were detected, Mch2α (1.7 kb) and Mch2β (1.4 kb), the former encoding the full-length Mch2 and the latter encoding a shorter isoform, possibly as a result of alternative splicing. Mch2α (caspase-6) encodes a 293-amino-acid protein with a predicted molecular mass of ~ 34 kDa. Mch2β contains a deletion corresponding to nucleotides 119–385 of the Mch2α sequence (amino acids 14–102) and encodes a 204-amino-acid protein with a predicted molecular mass of ~ 23 kDa. Mch2β lacks half of the p20 subunit and is probably catalytically inactive [81]. Expression of Mch2α, but not Mch2β, in insect cells results in the induction of apoptosis. Caspase-6 is a member of

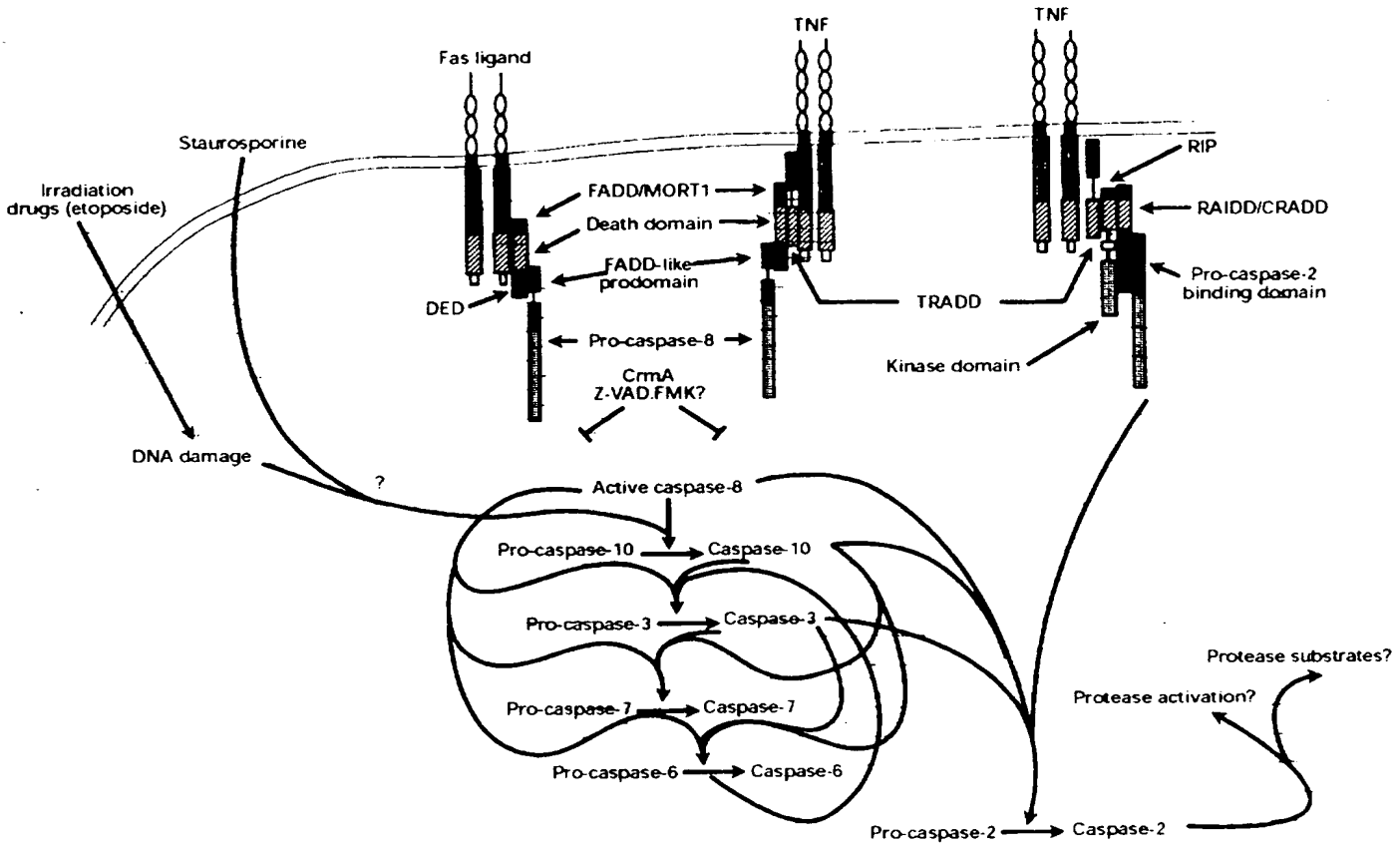


Figure 4 Hypothetical hierarchy of caspases

Apoptosis can be triggered by drugs (staurosporine), by DNA-damaging agents (etoposide or γ-irradiation) or by CD95 (Fas/APO-1) or tumour necrosis factor (TNF) interacting with their respective receptors. Both of these receptors transduce their apoptotic signals via intracellular C-terminal death domains (hatched boxes) which are involved in protein–protein interactions. Both CD95 and TNF result in the cleavage and activation of caspase-8, which can cleave all other known caspases. Caspase-10, which also cleaves all other known caspases, is activated by different stimuli via a CrmA-insensitive pathway, and may be activated following DNA damage (?). Activation of some caspases, such as caspase-6, results in their being able to cleave the caspase initially responsible for their formation, so setting up a protease amplification cycle [82]. Caspases may also be recruited to the death pathways by adapter molecules such as RAIDD/CRADD, which bind to RIP and to the prodomains of certain caspases such as pro-caspase-2 [101,102]. Part of this scheme, showing which caspase activates other caspases, is based on *in vitro* studies with purified enzymes, and requires confirmation from studies with intact cellular systems.

the CED-3 subfamily, showing high identity with caspase-3 (Figure 2a and Table 2).

Caspase-6, when expressed as a glutathione S-transferase fusion protein in *Escherichia coli*, autoprocesses and cleaves Ac-DEVD-AMC [although it is much less active ($\sim$ 150-fold) than caspase-3], but it does not cleave Ac-YVAD-AMC. Caspase-6 is also not readily inhibitable by Ac-DEVD.CHO [82]. Recombinant caspase-6 cleaves human PARP to give a smaller fragment than that seen in cells undergoing apoptosis, suggesting that caspase-6 does not play a major role in the cleavage of PARP [81]. Purified human recombinant caspase-3 cleaves ^{35}S-labelled pro-caspase-6 at three aspartate cleavage sites (Asp-23, Asp-179 and Asp-193), resulting in the formation of the large (p18) and small (p11) subunits of caspase-6 [82]. Initial cleavage appears to be at $D^{176}VVD\downarrow N^{180}$, resulting in a p13 subunit, which is then further cleaved at $T^{190}EVD\downarrow A^{194}$, resulting in the p11 subunit (Figure 3). Activation of pro-caspase-6 by caspase-3 results in an active enzyme that is capable of cleaving an artificially introduced lamin cleavage site (VEID$\downarrow$N) [82]. These data suggest that caspase-3 is activated prior to, and may be responsible for, the activation of caspase-6. However, these results are in apparent conflict with a recent study which identified and purified from hamster liver a homologue of caspase-6 that is capable of activating caspase-3 [83]. In addition, caspase-6 processes pro-caspase-3 at the $I^{172}ETD\downarrow S^{176}$ site between the large and small subunits, and this cleavage is blocked when Asp-175 is mutated to Ala [82]. Thus activation of caspase-3 can result in activation of pro-caspase-6 but, similarly, activation of pro-caspase-6 can also result in activation of caspase-3, resulting in a protease amplification cycle (Figure 4) [82]. Some caution must be exercised in the interpretation of such *in vitro* experiments, as purified or partially purified proteases may cleave substrates that they would not cleave *in vivo*, either because the ratio of enzyme to substrate would never reach such a level in the cell or because the enzyme may be located in a different subcellular environment from the substrate.

Mch3/ICE-LAP3/CMH-1 (CASPASE-7)

Caspase-7 was cloned independently in three different laboratories and named Mch3/ICE-LAP3/CMH-1 [84–86]. A member of the CED-3 subfamily, it is a 303-amino-acid protein with high

similarity to caspase-3 (Figure 2a and Table 2). An alternatively spliced isoform of caspase-7, which may act as a negative regulator of apoptosis, has been described [84]. Caspase-7 is constitutively expressed in many foetal and adult tissues, with lowest expression observed in the brain. Using a rabbit anti-peptide antibody, caspase-7 protein migrates at ~ 35 kDa and is detected to a variable extent in a number of cell lines, including Jurkat T cells, where it is localized diffusely to the cytoplasm and juxtamembrane structures [85], consistent with the suggestion that the death effector machinery resides in the cytoplasm rather than the nucleus [8]. Overexpression of full-length caspase-7 in the MCF7 breast carcinoma cell line does not induce apoptosis, whereas expression of a truncated derivative, lacking the 53 N-terminal amino acids corresponding to the putative prodomain, induces apoptotic cell death [85]. Bacterially expressed caspase-7, like caspase-3, preferentially cleaves PARP and the peptide substrate Ac-DEVD-AMC, but not Ac-YVAD-AMC or pro-IL-1β. The competitive peptide aldehyde inhibitor Ac-DEVD.CHO is a potent inhibitor of both caspase-3 and caspase-7, whereas Ac-YVAD.CHO and CrmA are poor inhibitors of both of these enzymes. As caspase-3 and caspase-7 are functionally similar and have similar substrate specificities [84], cleavage of PARP during apoptosis may be due to a combination of the action of both these caspases. Active caspase-7 is made up of two subunits, similar to other caspases [66] (Figure 3). Caspase-7 is activated to its catalytically active large subunit in intact cells undergoing apoptosis [62,85,87].

Following cleavage at Asp-198 and Asp-23, granzyme B activates pro-caspase-7 to a form that cleaves PARP to its signature fragment of ~ 85 kDa [66,88,89]. Using a caspase-7 protein with Asp-23 mutated to glutamate, it was demonstrated that removal of the prodomain by autoprocessing is not necessary for activity and that the p25/p12 enzyme is as active as the p20/p12 enzyme [89]. This suggested that the prodomain of caspase-7 may be involved in an as yet uncharacterized way in the regulation of caspase-7. *In vitro*, caspase-7 is a better substrate for granzyme B than is caspase-3. Pro-caspase-7 appears to exist as dimers or higher-order oligomers [89]. Incubation of Jurkat T cells with granzyme B together with a sublytic concentration of perforin results in the activation of endogenous caspase-7 prior to the induction of apoptosis. Together, these data suggest that caspase-7 may be an important intracellular effector of granzyme B-mediated apoptosis and cytotoxic T-lymphocyte-induced cell killing *in vivo*.

MACH/FLICE/Mch5 (CASPASE-8)

CD95 and the TNF receptor (TNFR-1; p55-R) are members of the TNF/nerve growth factor receptor family. Activation of these cell-surface cytokine receptors, either by their natural ligands or by agonistic antibodies, results in apoptosis. CD95 and TNFR-1 share a region of identity termed the 'death domain' that is required to signal apoptosis. Using a yeast two-hybrid system, three proteins were identified that bind to either the intracellular domains of CD95 and/or TNFR-1 through hetero-association of homologous regions found in these proteins. FADD/MORT1 (Fas-associating protein with death domain) [90,91] binds specifically to CD95, TRADD (TNFR-1-associated death domain protein) binds to TNFR-1 [92], and RIP (receptor-interacting protein) binds to both receptors [93]. Activation of CD95 initiates the association of at least four proteins (CAP1–CAP4), two of which (CAP1 and CAP2) have been identified as alternative forms of phosphorylated FADD/MORT1 [94]. A dominant-negative version of FADD, lacking the N-terminal death effector domain (DED), blocks the recruitment of the two other proteins (CAP3 and CAP4) to the death-inducing signalling complex [91], suggesting that these two proteins are downstream components of the CD95 signalling cascade. The region encompassing the 117 N-terminal amino acid residues of FADD can trigger apoptosis, and have been called DED [95].

Two groups independently identified a novel caspase, named MACH/FLICE (caspase-8), which contains both an active subunit with identity with the caspases and an N-terminal prodomain containing two domains with marked identity with the N-terminal DED of FADD/MORT1 [96,97] (Figure 3). Using nano-electrospray tandem MS, peptide sequences were obtained for CAP4 which, when used as sequence tags, identified a 3 kb cDNA that encodes a novel protein, caspase-8, of predicted molecular mass 55.3 kDa. The other group used a yeast two-hybrid screen to identify a cDNA clone with a novel sequence which binds to MORT1/FADD. This novel protein (MACH) occurs in multiple isoforms, most probably produced by alternative splicing, some of which contain a region with identity with the caspases. Northern blot analysis revealed a heterogeneity of caspase-8 transcripts which varied in amount and size in different human tissues. Few caspase-8 transcripts are detectable in testis, skeletal muscle and brain, with a relatively higher level of expression in peripheral blood leucocytes, consistent with a role for CD95-induced apoptosis in lymphocyte homoeostasis.

Caspase-8 contains two N-terminal stretches of approx. 70 amino acids that are apparently homologous to the DED of FADD. Residues 7–75 and 101–169 of caspase-8 share 39% identity (55% similarity) and 28% identity (55% similarity) respectively with the DED of FADD (residues 4–76) [97]. While the N-terminal portion of caspase-8 contains the FADD homology domains, the remainder of the protein is highly similar to the CED-3 subfamily of caspases (Figure 2a and Table 2). Instead of the active-site QACRG pentapeptide found in most caspases, caspase-8 contains the novel sequence QACQG. Caspase-8 associates with the DED of FADD. Granzyme B activates caspase-8 to an active protease which cleaves PARP to its characteristic signature fragment. Overexpression of caspase-8 results in apoptosis, and mutation of its catalytic cysteine residue abolishes its apoptotic potential. Expression of caspase-8 in the presence of isoforms with an incomplete caspase region resulted in little cell death. Similarly, these isoforms lacking a complete caspase region blocked cell death induced by CD95 and TNF, suggesting that the isoforms exert a dominant-negative effect and may be important in the regulation of apoptosis *in vivo*.

The marked heterogeneity of isoforms of caspase-8 compared with other caspases may provide a mechanism for some tissues or cells to protect themselves against CD95- or TNF-induced cell death [96]. These studies provide a critical link between activators and effectors of the cell death machinery. Oligomerization of the death domain of either CD95 or TNFR-1 allows recruitment of cytosolic adapter proteins to assemble a death-inducing signalling complex [98]. Thus CD95 (a cell death receptor) uses FADD (an adapter molecule) to interact physically with caspase-8 (a cytosolic protease) and initiate the apoptotic cascade (Figure 4). The precise mechanism by which recruitment of caspase-8 results in its activation is not known. It has been suggested that, in the latent state, the two DEDs of caspase-8 bind to each other, so preventing activation. The binding of FADD following the triggering of apoptosis by CD95 or TNF may cause a conformational change in the DED of FADD, so facilitating its binding to one of the DEDs of caspase-8, thereby disrupting the association of the two DEDs of caspase-8 and allowing its caspase domain to undergo autocatalytic activation [96,97].

Independently, Mch5 was cloned from Jurkat T cells, and its predicted sequence is almost identical with that of caspase-8 [66,96]. A small difference in the prodomain is revealed following sequence comparison of Mch5 and MACH/FLICE (G. M. Cohen, unpublished work). Bacterial expression of pro-caspase-8 generates a mature enzyme composed of two subunits, derived by processing of the proenzyme (Figure 3). Recombinant caspase-8 is able to process/activate all known caspases, including caspases-1 to -7 and caspases-9 and -10 [66,67], supporting the suggestion that it lies at the apex of an apoptotic cascade, at least from some stimuli such as CD95 or TNF [96,97].

ICE-LAP6/Mch6 (CASPASE-9)

Recently, two groups independently cloned a new member of the caspase family, ICE-LAP6/Mch6 (caspase-9) [82,99]. On searching the databases for genes related to that for caspase-7, a cDNA clone was identified that encodes a novel 416-amino-acid protein with a predicted molecular mass of ~ 46 kDa. Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3 (Table 2). The major difference between caspase-9 and other family members is the active-site pentapeptide QACGG, in which a Gly is found instead of the usual Arg (Table 1). Pro-caspase-9 contains a long N-terminal putative prodomain with high similarity to the prodomains of CED-3 and caspase-2. Northern-blot analysis revealed the presence of multiple mRNA species, suggestive of alternative splicing. High levels of expression of caspase-9 are found in the heart, testis and ovary. Overexpression of caspase-9, but not of a mutant in which the catalytic Cys was replaced with an Ala, induced apoptosis in MCF7 cells.

Procaspase-9 contains two potential processing sites between its large and small subunits, $P^{312}EPD\downarrow A^{316}$ and $D^{327}QLD\downarrow A^{331}$. The latter motif is similar to the DEVD↓G site in PARP, suggesting that caspase-9 may be activated by caspase-3, while the former motif may be a potential granzyme B cleavage site, as it contains an acidic residue in the P_3 position. Using *in vitro* mutagenesis, it was demonstrated that both caspase-3 and granzyme B activated pro-caspase-9, although to differently sized products. Asp-330 was the processing site for caspase-3, generating two products of molecular masses ~ 37 kDa (p37) and ~ 10 kDa (p10). Granzyme B cleaved procaspase-9 at both sites, with a marked preference for Asp-315 over Asp-330, generating an active enzyme capable of cleaving PARP to its signature fragment of ~ 85 kDa. In some, but not all, studies the prodomain of caspase-9 was removed [82,99]. The ability of caspase-3 to activate pro-caspase-9 suggests that the latter is downstream of caspase-3 and, as such, may be responsible for some of the later changes seen in cells undergoing apoptosis.

Mch4 (CASPASE-10)

Searching the databases of expressed sequence tags for sequences related to caspase-3 and caspase-6 led to the cloning from Jurkat T cells of a novel cDNA encoding a 479-amino-acid protein, Mch4 (caspase-10), with a molecular mass of ~ 55 kDa [66]. Caspase-10, a member of the CED-3 subfamily, is more closely related to caspase-8 than to any other caspase (Figure 2a and Table 2). Like caspase-8, caspase-10 has an active-site QACQG pentapeptide and also contains two FADD-like DEDs in its N-terminal domain, suggesting a possible role in CD95- or TNF-induced apoptosis. Mature caspase-10 is derived from a single-chain polypeptide proenzyme by cleavage at Asp-372 located between the large and small subunits (Figure 3). Northern blot analysis revealed that caspase-10 mRNA is present in most tissues, with lowest expression being observed in brain, kidney, prostate, testis and colon and higher levels in heart, liver and spleen. Recombinant caspase-10 is unusual in that it has a similar K_m for the cleavage of both Ac-DEVD-AMC and Ac-YVAD-AMC, but it is more similar to caspase-3 as it is potently inhibited by Ac-DEVD.CHO [66]. Granzyme B cleaves procaspase-10, lacking the N-terminal FADD-like domains, at $I^{369}EAD\downarrow A^{373}$. Purified recombinant caspase-10 processes all caspases, including pro-caspases-3, -7 and -10, whereas neither caspase-3 nor caspase-7 cleaves pro-caspase-10, suggesting that the latter is upstream of both caspase-3 and caspase-7 and lies at or near the apex of a cascade of proteases [66,67] (Figure 4).

GENERAL FEATURES OF THE CASPASES

Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (p20) and small (p10) subunits. In some cases these subunits are separated by a linker region of unknown function but which may be involved in regulation of the activation of the caspase. All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). The amino acids Cys-285 and His-237 involved in catalysis, and those involved in forming the P_1 carboxylate binding pocket in caspase-1 (Arg-179, Gln-283, Arg-341 and Ser-347), are conserved in all the other caspases, except for the conservative substitution of Thr for Ser-347 in caspase-8 (Figure 2b). This explains the absolute requirement for an Asp in the P_1 position. However, the residues that form the P_2 P_4 binding pocket are not well conserved, suggesting that they may determine the substrate specificities of the different caspases. It is evident from studies such as those with the caspase-1$^{-/-}$ and caspase-3$^{-/-}$ mice that not all caspases are required for cell death, and that some are more important than others. The importance of the tissue specificity of individual caspases is illustrated by the effects on the brains of caspase-3$^{-/-}$ mice [73]. Alternatively spliced isoforms of many caspases may in part regulate the activity of the full-length enzyme, either by acting as dominant inhibitors or by forming inactive heteromeric complexes [58,96]. A key role for a particular caspase in apoptosis has often been inferred from its overexpression resulting in the induction of this process. However, such overexpression may lead to the caspase cleaving substrates that it does not normally recognize. In addition, injection of other proteases such as trypsin or chymotrypsin into the cytoplasm of various cell types results in the induction of apoptosis [100].

Some caspases contain only a short prodomain (caspases-3, -6 and -7), whereas others contain long prodomains (caspases-1, -2, -4, -5, -8, -9 and -10) (Figure 3). The importance of the FADD-like prodomains of caspase-8 and possibly caspase-10 in directly linking CD95- and TNFR-1-mediated apoptosis has already been emphasized [96,97]. The significance of the other prodomains is not known, but they may be important in regulation of the activation of the caspases [43]. This possibility has been highlighted by the identification of a new adapter molecule, RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain)/CRADD (caspase and RIP adapter with death domain) [101,102]. RAIDD/CRADD comprises two main domains: an N-terminal domain which resembles and binds by a homophilic mechanism to the prodomain of caspase-2 and CED-3, and a C-terminal death domain that binds to RIP, a Ser/Thr kinase, which associates with CD95 and induces death [101]. The N-terminus of RAIDD/CRADD has significant identity with the prodomains of caspase-2 (residues 15–91: 31% identity), caspase-9 (residues 1–79: 28% identity) and CED-3

(residues 2–78; 24% identity), as well as with the C-terminus of a human inhibitor of apoptosis (IAP-1) [102]. Mutation of highly conserved residues in the N-terminal prodomains of caspase-2 and CED-3 abolishes their binding to RAIDD. RAIDD binds RIP, part of the TNFR-1 signalling complex, but not FADD or TRADD, except in the presence of RIP, when it can bind the latter. RIP recruits RAIDD/CRADD, which in turn recruits caspase-2, so creating a direct link to the effector caspases [101,102] (Figure 4). Interestingly, mature caspase-2 processes its own precursor, but not other currently known caspases [102]. Thus prodomains of some caspases may enable them to be recruited specifically to facilitate the execution of the cell death programme. It remains to be determined whether there is a family of RAIDD-like molecules that can recruit other caspases with long prodomains.

PROTEIN SUBSTRATES CLEAVED BY CASPASES DURING THE EXECUTION PHASE OF APOPTOSIS

During the execution phase of apoptosis, several proteins, including PARP, lamin B and histone H1, are cleaved [103]. The number of proteins identified as being cleaved during apoptosis is increasing rapidly. The caspases responsible for these reactions are indicated in Table 3; however, other caspases may also cleave these substrates. It is also not known which caspase(s) is/are responsible for cleavage under physiological conditions, or whether there is redundancy in the proteases for these cleavages. Some caspases show overlapping specificities for some substrates (caspase-3 and caspase-7 can both cleave PARP), whereas other caspases may have a unique substrate specificity (to date, caspase-6 is the only caspase known to cleave lamins). The biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology is often not known. Caspase-3 is responsible, either wholly or in part, for the proteolytic cleavage of a large number of substrates during apoptosis, including PARP, DNA-dependent protein kinase (DNA-PK), U1-70 kDa, heteronuclear ribonucleoproteins C1 and C2, sterol regulatory binding protein, D4-GDP dissociation inhibitor, huntingtin, and almost certainly retinoblastoma protein (Rb) [104–111] (Table 3). A common feature of all these substrates is the presence of a DXXD motif (Table 3), similar to that originally described in PARP [68]. Based on the cleavage site of PARP, Ac-DEVD-AMC was designed as a fluorimetric substrate for the measurement of caspase-3 activity. However, since caspase-7 also cleaves this substrate and this reaction is also potently inhibited by Ac-DEVD.CHO, it is likely that many substrates described as being cleaved by caspase-3 will also be found to be cleaved by caspase-7.

PARP

PARP is possibly the best characterized proteolytic substrate of caspases, being cleaved in the execution phase of apoptosis in many systems, including thymocytes, HL-60 cells and breast cancer cell lines [103,104]. Intact PARP (116 kDa) is cleaved to 24 kDa and 89 kDa fragments, representing the N-terminal DNA binding domain and the C-terminal catalytic domain of the enzyme respectively [104]. This possibly conserves the cellular NAD^+ and ATP normally required for PARP activity, thereby enabling the ATP to be utilized for the execution of apoptosis [7]. Cleavage of PARP may also interfere with its key homoeostatic function as a DNA repair enzyme [105]. PARP is cleaved at the sequence DEVD↓G by a protease activity resembling ICE (prICE), but not by ICE itself [68]. Although the cleavage of PARP is often a valuable indicator of apoptosis, its biological relevance, if any, is unclear, since PARP-null mice develop normally [112]. *In vitro*, other caspases, including caspases-2, -4, -6, -7, -8, -9 and -10, when added at high concentrations, can also cleave PARP or DEVD-AMC [80]. The physiological significance of this cleavage of PARP by these caspases is still under investigation. At the present time, it appears that caspase-3 and caspase-7 are primarily responsible for PARP cleavage during apoptosis.

DNA-PK

DNA-PK, an enzyme involved in DNA double-strand-break repair, possesses a 460 kDa catalytic subunit ($DNA\text{-}PK_{cs}$) and a DNA binding component Ku, which is a heterodimer of 70 and 80 kDa subunits. During apoptosis in several different systems, $DNA\text{-}PK_{cs}$, but not Ku, is degraded by a caspase with properties similar to those of caspase-3 [105,106]. Caspase-3, but not caspases-1, -4 or -6, cleaves purified $DNA\text{-}PK_{cs}$ to fragments of a similar size to those observed in cells undergoing apoptosis. Degradation of $DNA\text{-}PK_{cs}$ should lead to a decrease in the DNA repair capacity of the cell, so abolishing its key homoeostatic function and facilitating the characteristic DNA degradation associated with apoptosis [105,106].

Lamins

The proteolysis of lamins, the major structural proteins of the nuclear envelope, is observed in different cells undergoing apoptosis [68,87,113–116] and may be responsible for some of the observed nuclear changes, since inhibitors of lamin cleavage prevent some of these changes [115,117]. An *in vitro* model of apoptosis has been developed in which normal nuclei, exposed to cytosol from apoptotic cells, undergo many of the characteristic biochemical and morphological changes of nuclear apoptosis, including chromatin condensation, fragmentation and margination, internucleosomal cleavage of DNA, and proteolysis of PARP and lamins [68,118]. In this model, the lamin protease is clearly distinct from the PARP protease, cleavage of PARP being significantly more rapid than that of lamins and less sensitive to inhibition by either YVAD chloromethyl ketone or tosyl-lysylchloromethane [115]. Caspase-6 (Mch2) cleaves lamin A at a conserved VEID↓N sequence to give a fragment similar to that seen in apoptotic cells and extracts [119,120]. The site of cleavage is in a well conserved α-helical rod domain, which may disrupt lamin–lamin interactions as well as interactions of lamins with other nuclear components [120]. This lamin cleavage is readily inhibited by YVAD chloromethyl ketone. Under conditions where caspase-6 cleaves lamin A, caspases-1, -3 and -7 do not, suggesting that caspase-6 may be the major laminase in cells undergoing apoptosis [119,120]. Other, as yet untested, caspases may also cleave lamins, as may a Ca^{2+}-regulated serine protease [115,121]. Pro-caspase-6 is activated in cells undergoing apoptosis induced either by anti-CD95 antibody or by staurosporine, and caspase-6 functions downstream of Bcl-2 and Bcl-x_L [119]. In CD95-treated HeLa cells, lamin B is preferentially cleaved early in apoptosis prior to cleavage of lamins A and C and internucleosomal cleavage of DNA; this suggests that B- and A-type lamins may be cleaved by different caspases [122].

U1-70 kDa

The activity of the U1 small nuclear ribonucleoprotein particle, which is essential for the splicing of precursor mRNA, is dependent on both the RNA and protein components, including U1-70 kDa. In several systems, including CD95- and TNFR-

induced apoptosis, U1-70 kDa is cleaved early in the apoptotic process to a 40 kDa fragment [105,123]. Purified caspase-3 cleaves U1-70 kDa at a DGPD↓G site, similar to the reaction observed in apoptotic cells. In lysates from apoptotic cells, the cleavage of U1-70 kDa is potently inhibited by Ac-DEVD.CHO but not by Ac-YVAD.CHO, strongly suggesting that caspase-3 or possibly caspase-7 is the major caspase responsible for its cleavage in apoptosis. Cleavage of U1-70 kDa separates the RNA binding domain from the distal arginine-rich region of the molecule, which may have a dominant-negative effect on splicing; such inhibition of splicing would block cellular repair pathways dependent on new mRNA synthesis [105].

Fodrin

During the execution phase of apoptosis, a number of important plasma-membrane changes occur, resulting in the recognition and subsequent phagocytosis of the apoptotic cell either by a professional phagocyte or by a neighbouring cell. Cleavage of important cytoskeletal proteins, including actin [124,125], Gas2 [126] and fodrin (non-erythroid spectrin) [127–129], during apoptosis may induce cell shrinkage and membrane blebbing, and alter cell survival signalling systems. α-Fodrin, an abundant membrane-associated cytoskeletal protein, is rapidly and specifically cleaved during CD95- and TNF-induced apoptosis, and this appears to be related to the membrane blebbing. Initially it was proposed that fodrin is cleaved by calpain I [127], but the cleavage is probably due to a caspase [128,129]. Fodrin contains several potential caspase cleavage sites, including a DETD↓S site only nine amino acids away from the proposed calpain cleavage site, which may have led to the initial confusion [128]. Treatment of cells with Ac-DEVD.CHO protected them from CD95-induced apoptosis, but did not prevent the proteolysis of fodrin [128], suggesting that fodrin proteolysis can be uncoupled from apoptosis and that it is mediated by a caspase other than caspase-3. If correct, this is an intriguing observation, as it suggests that some caspases (those responsible for the cleavage of fodrin) may be activated and yet apoptosis may still be prevented. An alternative possibility is that Ac-DEVD.CHO may have inhibited only some features of the apoptotic phenotype, including those used to assess apoptosis in the particular study [128]. The inhibitory characteristics of the caspase responsible for fodrin proteolysis, i.e. readily inhibited by Ac-YVAD chloromethyl ketone and relatively insensitive to Ac-DEVD.CHO, are reminiscent of caspase-6.

Protein kinase C δ and Rb

Protein kinase C δ is also specifically cleaved during apoptosis to a catalytically active fragment by caspase-3, but not by caspases-2, -4, -5, -6 and -7. Protein kinase C δ is one of the few examples of a substrate that is cleaved by caspase-3 but not by caspase-7. Interestingly, overexpression of this fragment, but not of full-length protein kinase C δ or a kinase-inactive fragment, is associated with chromatin condensation, nuclear fragmentation and cell death, suggesting that the proteolytic activation of protein kinase C δ may contribute to certain features of the apoptotic phenotype [130,131].

Rb is an important mediator of cell cycle progression and regulation. Phosphorylation of Rb by cyclin-dependent kinases inactivates its growth-suppressive functions and drives cells through the cell cycle into mitosis. More recently, an anti-apoptotic function of Rb has also been described [132]. Several groups have described the cleavage of Rb during apoptosis [111,133,134]. Interestingly, different-sized cleavage products of Rb were observed in these studies, suggesting the possible involvement of different caspases. In one study, the cleavage site of Rb was identified as a DEAD↓G motif in the C-terminal region of the molecule, suggesting the likely involvement of caspase-3. The cleaved Rb failed to bind the regulatory protein Mdm2, which may lead to apoptosis due to inactivation of Rb functions [111].

INHIBITION OF CASPASES BY CrmA, Bcl-2 FAMILY MEMBERS, p35 AND PEPTIDE INHIBITORS

CrmA

When ectopically expressed, the *crmA* gene prevents apoptosis in a number of different systems [21,22,58,92,135–137] (Table 4). However, the ability of CrmA to inhibit apoptosis is clearly dependent on the stimulus used to induce it (Table 4). Thus there are CrmA-sensitive and -resistant pathways present in the same cell types. The recognition motif cleaved within CrmA is LVAD [138]. CrmA was originally described as a novel specific inhibitor of caspase-1 [45], but it is now apparent that it differentially inhibits different caspases. CrmA is a protease inhibitor that inhibits cell death, most probably by inhibiting one or more caspases. CrmA is a poor inhibitor of CED-3, caspase-2, caspase-3, caspase-7 and caspase-10, but an effective inhibitor of caspases-1, -4, -6 and -8 (Table 1) [58,65,67,83,84,135,138,139]. CrmA blocks both CD95- and TNFR-1-mediated cell death at much lower concentrations than are required for the inhibition of caspase-3 and caspase-7 [23,49,50,64,85]. Caspase-3 and caspase-7 are proteolytically activated following stimulation with CD95 or TNFR-1, but remain as zymogens in anti-CD95-treated CrmA-expressing cells, suggesting that CrmA inhibits a protease upstream of caspases-3 and -7 [85,140]. The effects on caspases-8 and -10 may be particularly important, as both of these caspases are capable of processing themselves and all other known caspases [67], and caspase-8 appears to be at the apex of the apoptotic cascade induced by CD95 or TNF [96,97]. Caspase-8 is very sensitive to inhibition by CrmA, whereas caspase-10 is only poorly inhibited, requiring a ~ 1000-fold greater concentration of CrmA to give the same inhibition [67]. Thus the sensitivity of CD95-induced apoptosis to inhibition by CrmA appears to be due to inhibition of caspase-8. Overexpression of caspase-8 results in apoptosis, which is inhibited by CrmA, suggesting that caspase-8 may be the physiological target of CrmA or, alternatively, that there is a downstream CrmA-

Table 4 Bcl-2 and CrmA regulate different pathways

Apoptosis induced in the same cells by different stimuli is often CrmA-sensitive or CrmA-resistant. The former pathways are frequently Bcl-2/Bcl-x_L-resistant, while the latter are Bcl-2/Bcl-x_L-sensitive. Abbreviations: NGF, nerve growth factor; ND, not determined; Ara-C, Cytarabine.

Treatment	Cells	CrmA-sensitive	Bcl-2/Bcl-x_L sensitive	References
Mouse ICE	Rat-1	+ + +	+ + +	21
CED-3	Rat-1	+	+	21
Caspase-2	Rat-1, HeLa	+	+ + +	58,135
NGF withdrawal	Dorsal ganglion neurons	+ +	+ +	22
CD95	Jurkat, BJAB, MCF7	+ + +		135,140
Staurosporine	Jurkat T cells		+ + +	140
CD95	Lymphoma cells	+ + +		136
γ-Irradiation	Lymphoma cells		+ + +	136
CD95	U937	+ + +	ND	137
Ara C	U937		+ + +	137
TRADD	HepG2, NIH 3T3	+ + +	–	92

sensitive protease. These results further support the suggestion that caspase-8 lies at the apex of the apoptotic cascade triggered by CD95 and TNF, and explain why CrmA is such a potent inhibitor of apoptosis resulting from these stimuli (Figure 4). In contrast, CrmA-insensitive pathways may involve caspase-10 or a closely related homologue as their most upstream caspase (Figure 4).

Bcl-2 family

Bcl-2 is the mammalian homologue of CED-9, which is a negative regulator of CED-3 [19]. Bcl-2 and related family members, such as Bcl-x_L, inhibit cell death induced by many stimuli [141,142]. Interestingly, many of the apoptotic pathways that are sensitive to inhibition by CrmA are relatively resistant to Bcl-2/Bcl-x_L, and vice versa (Table 4). These findings suggest the presence of a CrmA-sensitive (CD95)/Bcl-x_L-insensitive and a CrmA-insensitive/Bcl-x_L-sensitive pathway for the induction of apoptosis [137]. As discussed earlier, specific interactions of TRADD and FADD with an intracellular domain of TNFR-1 and CD95 respectively induce apoptosis, and both of these are inhibited by CrmA, whereas neither Bcl-2 nor the *E1B* gene product block TRADD-induced cell death [91,92]. The CrmA-sensitive pathways generally appear to involve plasma membrane receptor-mediated apoptosis, compatible with the inhibition of a specific caspase (such as caspase-8) being recruited to the cell membrane. Bcl-2/Bcl-x_L seems relatively ineffective at inhibiting this type of receptor-mediated apoptosis, but more efficacious than CrmA at inhibiting other forms of apoptosis.

Several recent studies suggest that Bcl-2 and Bcl-x_L exert their anti-apoptotic action at or before the processing of certain caspases to their catalytically active forms [140,143–145]. For example, overexpression of Bcl-2 or Bcl-x_L prevents staurosporine-induced cell death of Jurkat T cells and the processing of both caspase-3 and caspase-7, placing these negative regulators of apoptosis at or upstream of the processing of caspases-3 and -7 [140].

p35

The baculovirus *Autographa californica p35* gene product inhibits apoptosis in insects, mammals and nematodes, suggesting that it acts at a central and evolutionarily conserved part of the apoptotic pathway [24,138,146,147]. Purified recombinant p35 inhibits the activity of purified recombinant caspases, including caspases-1, -2, -3 and -4, and maximum inhibition is achieved at equimolar concentrations of p35 and the caspase. Cleavage of p35 by a caspase results in the formation of a caspase–p35 complex. The presence of such complexes *in vivo* would prevent the caspases from initiating the apoptotic cascade. The CED-3 cleavage site in p35 is DQMD↓G, which is required for protection against programmed cell death in the nematode. Interestingly, CrmA does not protect against cell death in the nematode unless its caspase-1 cleavage site (LVADC) is replaced by the CED-3 cleavage site in p35 [138].

Peptide inhibitors

In addition to the reversible inhibitors Ac-DEVD.CHO and Ac-YVAD.CHO, use of the irreversible tripeptide caspase inhibitor Z-VAD.FMK [benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone] has helped to elucidate the role of caspases in apoptosis, in particular in intact cellular systems. Z-VAD.FMK is a cell-permeable irreversible inhibitor of caspases whose permeability is facilitated by the presence of the benzyloxycarbonyl and OMe groups. Z-VAD.FMK is a potent inhibitor of apoptosis induced by a wide range of stimuli in a number of different systems, including thymocytes, hepatocytes, human monocytic THP.1 cells, Jurkat T cells, neuronal cells and *Drosophila melanogaster* [72,144,148–153]. Z-VAD.FMK also suppresses programmed cell death in the interdigital webs of developing mouse paws, suggesting that it may be of value in studying developmental cell death [72,154]. Z-VAD.FMK inhibits apoptosis at an early stage, as judged by its inhibition of all the ultrastructural features of apoptosis, i.e. PARP cleavage, processing of caspase-3, formation of large fragments and internucleosomal cleavage of DNA [71,72,149,150]. More recently we have shown that it inhibits the processing of caspases-2, -3, -6 and -7, suggesting that it inhibits a caspase at or near the apex of the apoptotic cascade [62]. In this regard, it is of interest that Z-VAD.FMK blocks apoptosis induced by caspase-8 [97]. Many, but not all, apoptotic cell deaths are inhibited by Z-VAD.FMK [155,156]. Whereas it completely inhibited apoptotic death induced by different stimuli in thymocytes [150,155], it had a variable effect on cell death in peripheral T cell blasts while complete sensitivity to another caspase inhibitor was maintained, suggesting the activation of different caspases at different stages of T-cell maturation [155]. Z-VAD.FMK did not inhibit Bax (a Bcl2 family member)-induced cell death, although it did inhibit certain features of the apoptotic phenotype, including cleavage of nuclear and cytosolic substrates and DNA fragmentation, suggesting that Bax-induced cell death may not require caspases [156]. *In vivo*, Z-VAD.FMK prevents the normally fatal liver damage induced by anti-CD95 injection [157]. Activation of both caspase-1-like and caspase-3-like protease activities is detected in liver lysates, as is the cleavage of caspase-3 to its p17 fragment. Repeated injections of Z-VAD.FMK results in the complete survival of all animals, with no histopathological signs of liver damage. The treatment with Z-VAD.FMK does not prevent the initial early small rise in ICE-like activity, as assessed by Z-YVAD-T-amino-4-trifluoromethylcoumarin cleavage; however, it does prevent processing of caspase-3 and the increase in caspase-3-like protease activity, as assessed by Z-DEVD-T-amino-4-trifluoromethylcoumarin cleavage. This and other observations suggest that non-toxic caspase inhibitors may have potential clinical applications in fulminant hepatitis arising from viral hepatitis [157] and various neurodegenerative disorders [5,154].

A HIERARCHY OF CASPASES

All caspases are cleaved at specific Asp residues, raising the possibility that some caspases sequentially activate others, so establishing a hierarchy of caspases. Such a model has been proposed in which caspase-8 has been termed an 'initiator' protease, which activates an 'amplifier' protease such as caspase-1, which in turn activates a 'machinery' protease such as caspase-3 or caspase-7 [158]. Much of the evidence for this, and the concept of a hierarchy of caspases, is based on *in vitro* data with recombinant enzymes; the limitations of such approaches have already been discussed. It is important, where possible, to determine if such a cascade of caspases occurs in cells undergoing apoptosis. PARP cleavage generally precedes lamin cleavage in cells undergoing apoptosis [68,87,115,116]. As caspase-3 and caspase-7 cleave PARP but not lamins, and caspase-6 cleaves lamins, this suggests that, in cells undergoing apoptosis, activation of caspase-3 and caspase-7 precedes activation of caspase-6 (Figure 4). In human monocytic tumour cells undergoing apoptosis, processing of caspases-2, -3, -6 and -7 was observed, although the precise sequence of activation of these caspases was not determined [62].

Different structural features, such as specific cleavage-site motifs, in the different caspases may give clues to their activities and to which caspase lies upstream of others (Table 1; Figures 2 and 3). The presence of a DXXD motif in caspases-2, -6 and -9 (Table 1) suggested that these three caspases may be activated by caspase-3, and this has been substantiated by *in vitro* experiments. A WXXD motif occurs in all three members of the ICE subfamily (Table 1), suggesting that they may be activated by the same caspase. Similarly, IXXD and LXXD motifs recur in different caspases (Table 1), supporting the notion that there may be a small number of key caspases that activate other family members. Good candidates for such critical caspases are caspase-8 and caspase-10, because they activate all other caspases and also contain FADD-like prodomains, which permit extensive protein–protein interactions [66,67,96,97]. Stimulation with either CD95 or TNF results in the recruitment and activation of caspase-8, supporting the hypothesis that it lies at the apex of the apoptotic cascade following specific receptor-mediated activation (Figure 4). Other types of receptor-mediated activation may involve other caspases, such as caspase-10. It has been proposed that caspase-10 may be involved in many CrmA-insensitive non-receptor-mediated cell deaths, as it is poorly inhibited by CrmA [67]. Once activated, some family members may then further activate the caspase that initially caused their activation, so setting up a caspase amplification cycle (Figure 4) [82]. The presence of CrmA-sensitive and -insensitive pathways also suggests that not all cell death pathways utilize the same caspases.

While many intracellular targets of the caspases have been recognized (Table 3), the critical cellular substrates leading to cell death have not yet been identified. The mechanisms by which caspases are regulated in cells in order to prevent their unwanted demise, and the possible role of apoptosis-inhibitory proteins, will be the subject of intense investigation. The long prodomains of some caspases, such as caspase-2, may enable them to be recruited by adapter molecules such as RAIDD/CRADD, so facilitating the execution of the cell death programme [101,102]. It remains to be determined whether there is a family of RAIDD/CRADD-like molecules that can recruit other caspases with prodomains.

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(57) Abstract

The present invention is directed to novel apoptosis polypeptides such as the Apop1, Apop2, and Apop3 proteins and related molecules which are involved in modulating apoptosis and to nucleic acid molecules encoding those polypeptides. Also provided herein are vectors and host cells comprising those nucleic acid sequences, chimeric polypeptide molecules comprising the polypeptides of the present invention fused to heterologous polypeptide sequences, antibodies which bind to the polypeptides of the present invention and to methods for producing the polypeptides of the present invention. Further provided by the present invention are methods for identifying novel compositions which modulate the biological activity of Apop1, Apop2, and Apop3, and the use of such compositions in diagnosis and treatment of disease.

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NOVEL APOPTOSIS PROTEINS

FIELD OF THE INVENTION

The invention relates to novel methods and compositions of apoptosis proteins, collectively termed "Apop proteins", and nucleic acids encoding them. The invention further relates to methods of screening for bioactive agents that bind to and modulate Apop protein function for the diagnosis and treatment of disease.

BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death, is a highly ordered, genetically controlled process which plays a vital role in both healthy and disease states, including embryogenesis, tissue homeostasis and remodeling, cancer, autoimmune disorders, viral infections, and certain degenerative disorders.

The death domain of TNF receptor-1 (TNFR1) triggers distinct signaling pathways leading to apoptosis and activation of the NF-κB transcription factor through its interaction with the C-terminal death domain of TRADD, a 34 kDa cytoplasmic protein [see Hsu et al., Immunity 4:387-96 (1996)]. TRADD interacts strongly with RIP (receptor-interacting protein; Stanger et al., Cell 81:513-23 (1995), a 74 kDa serine-threonine kinase that with a C-terminal death domain involved in apoptosis; RIP also activates NF-κB. A second RIP protein, RIP2 or RICK [see McCarthy et al., J. Biol. Chem. 273:16968 (1998) and Inohara et al., J. Biol. Chem. 273:12296 (1998)] also contains a death domain and activates NF-κB.

A characteristic feature of apoptosis is activation of a cascade of cytoplasmic proteases that results in the cleavage of selected target proteins. ICE (interleukin 1 beta-converting enzyme) family proteases, also known as caspase proteases, initiate the active phase of apoptosis by degrading specific structural, regulatory, and DNA repair proteins within the target cell [Lazebnik et al., Nature 371:346-7 (1994); Casciola-Rosen et al., J. Biol. Chem. 269:30757-60 (1994)]. For example, a RIP-like kinase, termed CARDIAK/RICK or RIP2 [see Thome et al., Current Biol. 8:885-88 (1998); McCarthy et al., J. Biol. Chem. 273:16968-75 (1998); Inohara et al., J. Biol. Chem. 273:12296-300 (1998)] has been shown to associate with caspase-1. These caspases are related to the *C. elegans* cell death gene product. Caspases are cysteine proteases that display aspartate specificity, and have been shown by a number of researchers to be crucial to apoptotic pathways. For a review, see Cryns et al., Genes & Development 12:1551-70 (1998). The natural substrates of the caspases are key regulatory and

structural proteins, including protein kinases and proteins involved in DNA repair and cytoskeletal integrity.

There are a number of inhibitors of apoptosis (IAPs) that have been identified. Originally identified in baculoviruses, IAPs suppress the host cell death response, thereby allowing survival and propagation of the virus. To date, there are five human IAPs identified, which when expressed in human cells can inhibit apoptosis induced by a variety of stimuli. In addition, the IAPs have been shown to be fairly selective, with different pathways and/or enzymes being inhibited. Human XIAP, cIAP1 and cIAP2 are direct inhibitors of at least two caspase family members, caspase-3 and caspase-7.

Accordingly, the proteins involved in apoptosis and its regulation are of paramount interest, and it is an object of the invention to provide novel apoptosis proteins, herein termed Apop proteins, and in particular Apop1, Apop2, and Apop3 proteins and related molecules. It is a further object of the invention to provide recombinant nucleic acids encoding Apop proteins, and expression vectors and host cells containing the nucleic acid encoding them. A further object of the invention is to provide methods for screening for antagonists and agonists of Apop proteins.

SUMMARY OF THE INVENTION

According to the objects outlined above, the present invention provides recombinant nucleic acids encoding Apop proteins, and in particular Apop1, Apop2 and Apop3 proteins, that are at least about 85% identical to the amino acid sequence depicted in Figure 2, Figure 4, and Figure 6, respectively. Similarly provided are recombinant nucleic acids at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 or their complements. Expression vectors and host cells comprising the nucleic acids are also included.

In a further aspect, the invention provides methods of making Apop proteins, comprising providing a cell comprising an Apop protein encoding nucleic acid and subjecting the cell to conditions which allow the expression of Apop proteins.

In an additional aspect, the invention provides recombinant Apop proteins, that are at least about 85% identical to the amino acid sequences depicted in Figures 2, 4, and 6, respectively, and antibodies that will bind to the Apop1 proteins, Apop2 proteins and Apop3 proteins.

In a further aspect, the invention provides monoclonal and polyclonal antibodies binding to the apoptosis proteins Apop1, Apop2, and Apop3.

In a further aspect, the present invention provides methods for screening for a bioactive agent capable of binding to an Apop protein. The method comprises combining a candidate bioactive agent and an Apop protein and determining the binding of the candidate agent to the Apop protein.

In an additional aspect, the present invention provides methods for screening for agents capable of interfering with the binding of Apop3 and RIP. The methods comprise combining an Apop3 protein, a candidate bioactive agent and a RIP protein, and determining the binding of the Apop3 protein and the RIP protein.

In an additional aspect, the present invention provides methods for screening for agents capable of interfering with the binding of Apop1 and XIAP or Apop2 and XIAP. The methods comprise combining an Apop1 protein or Apop2 protein, a candidate bioactive agent and an XIAP protein, and determining the binding of the Apop1 protein and the XIAP protein or Apop2 protein and the XIAP protein.

In an additional aspect, the invention provides methods for screening for an bioactive agent capable of modulating the activity of an Apop protein. The method comprises the steps of adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an Apop protein and determining the effect of the candidate bioactive agent on apoptosis.

In one aspect, a method for screening for a bioactive agent comprises providing a cell that expresses an expression profile gene selected from the group consisting of the expression profile genes set forth in Figures 1, 3, and 5.

In another aspect, a method for screening for a bioactive agent, comprises providing a cell that expresses an expression profile protein selected from the group consisting of the expression profile proteins having the amino acid sequence set forth in Figures 2, 4, and 6.

In another aspect, the invention provides a biochip comprising recombinant nucleic acids encoding Apop proteins. The recombinant nucleic acids, encoding Apop proteins and bound to the biochip, may be at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 or their complements.

Other aspects of the invention will become apparent to the skilled artisan by the following description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence of human Apop1. The putative translation start codon (ATG) and translation termination codon (TGA) are underlined.

Figure 2 depicts the amino acid sequence of human Apop1.

Figure 3 depicts the nucleotide sequence of human Apop2. The putative translation start codon (ATG) and the translation termination codon (TGA) are underlined.

Figure 4 depicts the amino acid sequence of human Apop2.

Figure 5 depicts the nucleotide sequence of human Apop3.

Figure 6 depicts the amino acid sequence of human Apop3.

Figure 7 depicts a schematic representation of Apop3 truncation mutants. The shaded area depicts the kinase homology domain. Apop3(K50D) has a K to D mutation at amino acid 50. WT, wild-type Apop3, i.e., the full-length protein.

Figure 8 depicts a summary of binding results of Apop3 to RIP in the yeast two hybrid system. ++++, very strong interaction; +++, strong interaction; +, detectable interaction; -, no detectable interaction.

Figure 9 depicts the activation of apoptosis by Apop3 in Phoenix-A cells. Empty vector (Vector), Apop3, or Apop3 (1-436) (3 μg) was co-transfected with pGDB (1 μg) into Phoenix-A cells. Hoechst stained apoptotic Phoenix-A cells were examined and counted by fluorescence microscopy. The data are expressed as percentage of apoptotic cells among the total number of cells counted.

Figure 10 depicts the activation of NFκB by Apop3 in Phoenix-A cells. NFκB reporter activity was performed by transiently co-transfecting Phoenix-A cells with the indicated Apop3 expression vectors (3 μg), NFκB–dependent luciferase reporter plasmid (1 μg), and Renilla Luciferase expression vector (0.13 μg). Calcium phosphate precipitation method was used for transfection. The level of expressed tagged proteins was also monitored by Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel apoptosis proteins and nucleic acids which were initially identified using a yeast two-hybrid screening method. Traditionally, protein-protein interactions were

evaluated using biochemical techniques, including chemical cross-linking, co-immunoprecipitation and co-fractionation and -purification. Recently genetic systems, including the "yeast two-hybrid system" have been described to detect protein-protein interactions. The basic system requires a protein-protein interaction between a "bait protein" and a "test protein" in order to turn on transcription of a reporter gene. See Fields et al., Nature 340:245 (1989); Vasavada et al., Proc. Natl. Acad. Sci. U.S.A. 88:10686 (1991); Fearon et al., Proc. Natl. Acad. Sci. U.S.A. 89:7958 (1992); Dang et al., Mol. Cell. Biol. 11:954 (1991); Chien et al., Proc. Natl. Acad. Sci. U.S.A., 88:9578 (1991); U.S. Patent Nos. 5,283,173, 5,667,973, 5,468,614, 5,525,490, and 5,637,463; and U.S. patent application S.N. 09/050,863. In particular Apop1 and Apop2 were identified using XIAP as the "bait protein". Apop3 was identified using RIP as the "bait protein".

Thus, the present invention provides novel apoptosis proteins, termed Apop proteins and nucleic acids encoding them. Unless otherwise explicitly stated herein, the terms "Apop", "Apop protein" or grammatical equivalents thereof include Apop1 proteins, Apop2 proteins, and Apop3 proteins, the wild-type amino acid sequences of which are depicted in Figure 2, Figure 4, and Figure 6, respectively. Similarly, the terms "Apop nucleic acid", "Apop DNA", "Apop nucleotide sequence" or grammatical equivalents thereof include nucleic acids which encode Apop1 proteins, Apop2 proteins and Apop3 proteins, the wild-type nucleic acid sequences of which are depicted in Figure 1, Figure 3, and Figure 5, respectively.

In a preferred embodiment, the Apop proteins are from vertebrates and more preferably from mammals, including rodents (rats, mice, hamsters, guinea pigs, etc.), primates, farm animals (including sheep, goats, pigs, cows, horses, etc) and in the most preferred embodiment, from humans. However, using the techniques outlined below, Apop proteins from other organisms may also be obtained.

An Apop protein of the present invention may be identified in several ways. "Protein" in this sense includes proteins, polypeptides, and peptides. An Apop nucleic acid or Apop protein is initially identified by substantial nucleic acid and/or amino acid sequence homology to the sequences shown in Figures 1, 2, 3, 4, 5, and 6. Such homology can be based upon the overall nucleic acid or amino acid sequence.

As used herein, a protein is an "Apop protein" if the overall homology of the protein sequence to the amino acid sequences shown in Figures 2, 4, and 6 is preferably greater than about 75%, more preferably greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.

Homology in this context means sequence similarity or identity, with identity being preferred. This homology will be determined using standard techniques known in the art, including, but not limited to, the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc Natl. Acad. Sci. U.S.A. 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), or the Best Fit sequence program described by Devereux et al., Nucl. Acid Res. 12:387-95 (1984), preferably using the default settings, or by inspection.

In a preferred embodiment, similarity is calculated by FastDB based upon the following parameters: mismatch penalty of 1.0; gap size penalty of 0.33, joining penalty of 30.0 ("Current methods in Comparison and Analysis", Macromolecule Sequencing and Synthesis, selected methods and Applications, pp. 127-149 (1998), Alan R. Liss, Inc.). Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, J. Mol. Evol. 35:351-60 (1987); the method is similar to that described by Higgins and Sharp CABIOS 5:151-3 (1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

An additional example of a useful algorithm is the BLAST algorithm, described in Altschul et al., J. Mol. Biol. 215: 403-410 (1990) and Karlin et al., Proc. Natl. Acad. Sci. U.S.A. 90:5873-87 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology 266:460-480 (1996); http://blast.wustl/edu/blast/ README.html]. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the coding sequence of the Apop proteins (see Figures 1, 3, and 5). A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

An additional useful algorithm is gapped BLAST as reported by Altschul et al., Nucl. Acid Res. 25:3389-3402 (1997). Gapped BLAST uses BLOSUM-62 substitution scores; threshold *T* parameter set to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of *k* a cost of 10+*k*; X_u set to 16, and X_g set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to ~22 bits.

The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein sequences shown in Figures 2, 4, and 6 it is understood that the percentage of homology will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Thus, for example, homology of sequences shorter than that shown in Figures 2, 4, and 6, as discussed below, will be determined using the number of amino acids in the shorter sequence.

In a preferred embodiment, the Apop protein binds to an IAP protein. Inhibitor-of-apoptosis (IAP) proteins are a novel family of anti-apoptotic proteins that were first identified in baculoviruses. Members of the human IAP family include, e.g., NAIP, HIAP-1 (human inhibitor of apoptosis protein-1), HIAP-2 (human inhibitor of apoptosis protein-2), c-IAP-1, c-IAP-2, and XIAP. They are thought to inhibit cell death via direct inhibition of caspases. Indeed, some IAP family proteins, e.g., c-IAP-1, c-IAP-2 and XIAP can bind to and inhibit the distal cell death proteases, caspases-3 and -7 (Roy et al., EMBO J., 16:6914-25 (1997); Takahashi et al., J. Biol. Chem. 273:7787-90 (1998)). Although IAPs are highly conserved through evolution, the mechanisms by which they interfere with the apoptotic cell death are not clear. There may be a number of unknown protein substrates for IAPs and agonistic or antagonistic proteins modulating the activity of IAPs.

In order to identify novel proteins interacting with IAPs, in particular with XIAP, we employed the yeast two-hybrid screening system. Using XIAP as the "bait protein" we identified two novel proteins interacting with XIAP, named Apop1 and Apop2.

In a preferred embodiment, the Apop protein is an Apop1 protein. The nucleotide sequence of Apop1 is depicted in Figure 1 and the encoded protein in Figure 2. A significant portion of the Apop1 nucleic

acid and the encoded Apop1 protein has identity to human cathepsin B precursor protein and the corresponding DNA [Ritonja et al., FEBS Lett. 181:169-172 (1985); Chan et al., Proc Natl. Acad. Sci. U.S.A. 83:7721-7725 (1986); Fong et al:, Proc Natl. Acad. Sci. U.S.A. 83:2909-2913 (1986); Moin et al., Biochem. J. 285:427-434 (1992); Cao et al., Gene 139:163-169 (1994)]. Cathepsin B is a lysosomal thiol proteinase that may have additional extralysosomal functions. In particular, nucleotides 364 to 716 of the Apop1 nucleotide sequence depicted in Figure 1 have >99% (352/353 nucleotide residues) identity to human cathepsin B (CTSB) mRNA (GenBank accession numbers NM_001908 and M14221). Likewise, on the amino acid level, the Apop1 amino acid sequence depicted in Figure 2, shows 100% (109/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number P07858) and >99% (108/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number NP_001899). However, the remainder of the Apop1 protein and nucleic acid do not exhibit any homology to the known sequences. Thus the Apop1 nucleic acid sequence of the present invention may represent an alternatively spliced cathepsin B mRNA transcript and the encoded protein may have other biological activities when compared to cathepsin B.

On the nucleic acid level, there are a number of reported sequences including EST99543 and EST41374 that have homology to Apop1.

In a preferred embodiment, the Apop protein is an Apop2 protein. The nucleotide sequence of Apop2 is depicted in Figure 3 and the encoded protein in Figure 4. Apop2 nucleic acid and the encoded Apop2 protein have identity to human cathepsin F precursor protein and the corresponding DNA [Wang et al., J. Biol. Chem. 273:32000-8 (1998); Nagler et al., Biochem. Biophys. Res. Commun. 257:313-8 (1999); Santamaria et al., J. Biol. Chem. 274:13800-9 (1999); Wex et al., Biochem. Biophys. Res. Commun. 259:401-7 (1999); GenBank accession numbers AF071748, NM_003793, AF088886, AJ007331, and AF132894]. Cathepsin F is a novel papain-like cysteine proteinase, synthesized as a precursor protein, including a hydrophobic signal sequence, a pro-domain, and a catalytic region [Santamaria et al., J. Biol. Chem. 274:13800-9 (1999)]. Surprisingly, Apop2 has strong homology to several other cathepsins. Protein sequence comparison revealed 58% homology with cathepsin W; about 42-43% with cathepsin L, K, S, H, and O; and 38% with cathepsin B [Wang et al., J. Biol. Chem. 273:32000-8 (1998)]. The identification of Apop2 (cathepsin F) by binding to XIAP, represents the first time that a protein containing cathepsin homology has been shown to play a role in apoptosis.

On the nucleic acid level, there are a number of reported EST sequences that comprise portions of the Apop2 nucleic acid. These include EST43911/AA38898, yp01g08.r1/H39591, and EST91038/

AA378321. In addition, there are several areas of homology to known cDNAs, including: 88% identity to a mouse mammary gland cDNA; (AA475592), 95% identity to a human cDNA clone (H15748), and a 88% identity to a mouse mammary gland cDNA (AA958896).

In a preferred embodiment, the Apop protein is an Apop3 protein. Using RIP as the "bait protein" in a yeast two-hybrid screening, Apop3 was identified. The nucleotide sequence of Apop3 is depicted in Figure 5 and the encoded protein in Figure 6. The human Apop3 appears to be expressed in heart, liver, pancreas, placenta and lung, but either weakly or not at all in brain. Apop3 has also been termed "RIP3" [see Yu et al., Current Biology 9:539-42 (1999); Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. The N-terminal portion of Apop3, in particular amino acid residues 1-274, has homology to the kinase domain of RIP (34% identity and 60% similarity) and RIP2 (31% identity and 58% similarity) [see Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. However, the C-terminal portion of Apop3 has no significant homology to any known proteins. Accordingly, Apop3 proteins may be identified in one aspect by significant homology to areas other than the kinase domain. This homology is preferably greater than about 60%, with greater than about 70 or 75% being particularly preferred and greater than about 80% being especially preferred. In some cases the homology will be greater than about 90 to 95 or 98%.

In addition, an Apop3 protein preferably also has significant homology to the kinase domain as described herein. This homology is preferably greater than about 75%, with greater than about 80% being particularly preferred and greater than about 85% being especially preferred. In some cases the homology will be greater than about 90 to 95 or 98%.

Apop proteins of the present invention may be shorter or longer than the amino acid sequences shown in Figures 2, 4, and 6. Thus, in a preferred embodiment, included within the definition of Apop proteins are portions or fragments of the sequences depicted herein. Portions or fragments of Apop proteins are considered Apop proteins if a) they share at least one antigenic epitope; or b) have at least the indicated homology; or c) preferably have Apop biological activity, e.g., including, but not limited to kinase activity, cell death activity, binding to XIAP or RIP, etc.

Well-known methods, such as in vitro manipulations of nucleic acids or PCR (polymerase chain reaction) are known in the art and are routinely used to generate portions or fragments of a desired nucleic acid sequence which then encodes a portion or a fragment of the desired protein. Generally, such portions of an Apop protein comprise at least 10%, preferably at least 20%, more preferably at least 25%, even more preferably at least 30% and most preferably at least 50% of the Apop protein.

In some embodiments, a portion or fragment of an Apop protein will comprise as much as 60 to 90 or 95% of the respective Apop protein.

In one embodiment, the portion or fragment of the respective Apop protein is contiguous to the Apop protein from which it is obtained. In another embodiment, the portion or fragment of the respective Apop protein is not contiguous to the Apop protein from which it is derived. In this embodiment, two or more parts of the Apop protein, for example, a fragment obtained from the N-terminus and a fragment obtained from the C-terminus are linked, whereby internal sequences are deleted.

In a preferred embodiment, the Apop proteins are derivative or variant Apop proteins. That is, as outlined more fully below, the derivative Apop peptide will contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the Apop peptide.

In addition, as is more fully outlined below, Apop proteins can be made that are longer than those depicted in Figures 2, 4, and 6, for example, by the addition of epitope or purification tags, the addition of other fusion sequences, etc.

Apop proteins may also be identified as being encoded by Apop nucleic acids. Thus, Apop proteins are encoded by nucleic acids that will hybridize to the sequence depicted in Figures 1, 3, and 5 or its complement, as outlined herein.

In one preferred embodiment, the Apop proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to Apop proteins, which are useful as described herein. The terms "Apop antibodies", "antibodies binding to Apop" or grammatical equivalents thereof include antibodies binding to Apop1 proteins, Apop2 proteins, and Apop3 proteins.

Apop antibodies usually are generated with an Apop protein having the amino acid sequence depicted in Figures 2, 4, and 6. In a preferred embodiment, Apop proteins corresponding to a portion or fragment of an Apop protein of which the amino acid sequence is depicted in Figures 2, 4, and 6, are used to generate antibodies. Methods for the preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988). When the Apop protein is used to generate antibodies, the Apop protein must share at least one epitope or determinant with the full length protein shown in Figures 2, 4, and 6. By "epitope" or "determinant" herein is meant a

portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Apop3 protein will be able to bind to the full length protein.

In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity to other proteins. The term "antibody" includes antibody fragments, as are known in the art, including Fab Fab_2, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. The term "antibody" further comprises polyclonal antibodies and monoclonal antibodies, which can be agonist or antagonist antibodies.

The Apop antibodies of the invention specifically bind to Apop proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a binding constant in the range of at least 10^{-4}- 10^{-6} M^{-1}, with a preferred range being 10^{-7} - 10^{-9} M^{-1}.

In a preferred embodiment, an Apop protein of the present invention may be identified by its immunological activity., i.e., its ability to bind to an antibody specific for an epitope found within a protein comprising the amino acid sequence depicted in Figures, 2, 4, and 6. The term "immunological activity" means the ability of the protein to cross react with an antibody which is specific for the protein comprising the amino acid sequence depicted in Figures 2, 4, and 6, i.e., an Apop protein antibody. Accordingly, a protein is an Apop protein, if the protein displays the immunological activity of a protein comprising the amino acid sequence depicted in Figures 2, 4, and 6.

In a preferred embodiment, Apop antibodies are provided. The antibodies may be polyclonal or monoclonal. In a preferred embodiment, the antibodies to Apop are capable of reducing or eliminating the biological function of Apop, as is described below. That is, the addition of anti-Apop antibodies (either polyclonal or preferably monoclonal) to Apop (or cells containing Apop) may reduce or eliminate the Apop activity. Generally, at least a 25% decrease in activity is preferred, with at least about 50% being particularly preferred and about a 95-100% decrease being especially preferred.

Monoclonal antibodies are directed against a single antigenic site or a single determinant on an antigen. Thus monoclonal antibodies, in contrast to polyclonal antibodies, which are directed against multiple different epitopes, are very specific. Monoclonal antibodies are usually obtained from the supernatant of hybridoma culture (see Kohler and Milstein, Nature 256:495-7 (1975); Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988).

In a preferred embodiment, the antibodies to Apop proteins are humanized. Using current monoclonal antibody technology one can produce a humanized antibody to virtually any target antigen that can be identified [Stein, Trends Biotechnol. 15:88-90 (1997)]. Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *supra*; Riechmann et al., *supra*; and Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Additional examples of humanized murine monoclonal antibodies are also known in the art, e.g., antibodies binding human protein C [O'Connor et al., Protein Eng. 11:321-8 (1998)], interleukin 2 receptor [Queen et al., Proc. Natl. Acad. Sci., U.S.A. 86:10029-33 (1989)], and human epidermal growth factor receptor 2 [Carter et al., Proc. Natl. Acad. Sci. U.S.A. 89:4285-9 (1992)]. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991); Marks et al., J. Mol. Biol. 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol. 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10:779-783 (1992); Lonberg et al. Nature 368:856-859 (1994); Morrison, Nature 368:812-13 (1994); Fishwild et al., Nature Biotechnology 14:845-51 (1996); Neuberger, Nature Biotechnology 14:826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13:65-93 (1995).

In a preferred embodiment, Apop nucleic acids are provided. An Apop nucleic acid of the present invention may be identified by its sequence identity to the nucleotide sequence depicted in Figures 1, 3, or 5 and may be referred to as having some "percent (%) sequence identity to all or a portion of the nucleotide sequence depicted in Figures 1, 3, or 5. Sequence identity, when referring to nucleic acid, means that the sequences being compared have nucleotides at corresponding positions which are identical. The sequence identity of an Apop nucleic acid is commensurate with the sequence identity of Apop proteins but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the sequence identity for Apop nucleic acids may differ from the sequence identity for Apop proteins. Thus, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence. Thus the homology of the nucleic acid sequence as compared to the nucleic acid sequences of Figures 1, 3, and 5 is preferably greater than 75%, more preferably greater than about 80%, particularly greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.

In a preferred embodiment, an Apop nucleic acid encodes an Apop protein. As will be appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the Apop proteins of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the Apop.

In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequences shown in Figures 1, 3, and 5 or their complements are considered an Apop gene. High stringency conditions are known in the art; see for example Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989) and Ausubel et al., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., 1995), both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Sambrook et al., *supra*, Ausubel et al., *supra*, and Tijssen, *supra*.

The Apop proteins and nucleic acids of the present invention are preferably recombinant. As used herein, and defined below, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences depicted in Figures 1, 3, and 5 also include the complement of these sequences. By the term "recombinant

nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated Apop nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e. using the in vivo cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of an Apop protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of a inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below.

Also included within the definition of Apop proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the Apop protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the recombinant DNA in cell culture as outlined above. However, variant Apop protein fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the Apop protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue,

although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed Apop variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of Apop protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the Apop protein are desired, substitutions are generally made in accordance with the following chart:

Chart I

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the Apop proteins as needed. Alternatively, the variant may be designed such that the biological activity of the Apop protein is altered. For example, glycosylation sites may be altered or removed. Similarly, e.g., mutations within the cysteine protease domain of Apop1 or Apop2 or within the kinase domain of Apop3 may be made.

Covalent modifications of Apop polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of an Apop polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of an Apop polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking Apop to a water-insoluble support matrix or surface for use in the method for purifying anti-Apop antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman &

Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the Apop polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apop polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence Apop polypeptide.

Addition of glycosylation sites to Apop polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apop polypeptide (for O-linked glycosylation sites). The Apop amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apop polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the Apop polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apop polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of Apop comprises linking the Apop polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Apop polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising an Apop polypeptide fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, such a chimeric molecule comprises a fusion of an Apop polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the Apop polypeptide. The presence of such epitope-tagged forms of an Apop polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apop polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of an Apop polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

Also included with the definition of Apop protein are other Apop proteins of the Apop family, and Apop proteins from other organisms, which are cloned and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related Apop proteins from humans or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the unique areas of the Apop nucleic acid sequence. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain inosine as needed. The conditions for the PCR reaction are well known in the art.

In another preferred embodiment, new members within an Apop family are identified within the different cells or tissues of the same organism. The amino acid sequences of individual members are aligned and analyzed as described above. Amino acid sequences which are identical among those family members are used to design PCR primers. These PCR primers take the degeneracy of the genetic code into consideration and thus, may e.g., incorporate inosine at the third codon position. Similarly, PCR primers are designed based on the alignment and analysis of the respective nucleic acid sequences. Thus, PCR primers are designed to bind to conserved nucleotide sequences shared

by different members of the Apop family. In this embodiment, nucleotide sequences for all members of the Apop family, i.e., known and unknown members, sharing the respective nucleotide sequences to which the PCR primers bind, are generated by PCR. Subsequent subcloning and DNA analysis of the generated DNA fragments, as is well known in the art and e.g. described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989) and Ausubel et al., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., 1995) identifies known and/or new members of the Apop family.

Once the Apop nucleic acid is identified, it can be cloned and, if necessary, its constituent parts recombined to form the entire Apop nucleic acid. Once isolated from its natural source, e.g., contained within a plasmid or other vector or excised therefrom as a linear nucleic acid segment, the recombinant Apop nucleic acid can be further-used as a probe to identify and isolate other Apop nucleic acids. It can also be used as a "precursor" nucleic acid to make modified or variant Apop nucleic acids and proteins.

Using the nucleic acids of the present invention which encode an Apop protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the Apop protein. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the Apop protein, as will be appreciated

by those in the art; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the Apop protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

Promoter sequences include constitutive and inducible promoter sequences. The promoters may be either naturally occurring promoters, hybrid or synthetic promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

In addition, the expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors and appropriate selection and screening protocols are well known in the art and are described in e.g., Mansour et al., *Cell*, 51:503 (1988) and Murray, *Gene Transfer and Expression Protocols, Methods in Molecular Biology, Vol. 7* (Clifton: Humana Press, 1991).

In addition, in a preferred embodiment, the expression vector contains a selection gene to allow the selection of transformed host cells containing the expression vector, and particularly in the case of mammalian cells, ensures the stability of the vector, since cells which do not contain the vector will generally die. Selection genes are well known in the art and will vary with the host cell used. By "selection gene" herein is meant any gene which encodes a gene product that confers resistance to a selection agent. Suitable selection agents include, but are not limited to, neomycin (or its analog G418), blasticidin S, histinidol D, bleomycin, puromycin, hygromycin B, and other drugs.

In a preferred embodiment, the expression vector contains a RNA splicing sequence upstream or downstream of the gene to be expressed in order to increase the level of gene expression. See Barret et al., Nucleic Acids Res. 1991; Groos et al., Mol. Cell. Biol. 1987; and Budiman et al., Mol. Cell. Biol. 1988.

A preferred expression vector system is a retroviral vector system such as is generally described in Mann et al., Cell, 33:153-9 (1993); Pear et al., Proc. Natl. Acad. Sci. U.S.A., 90(18):8392-6 (1993); Kitamura et al., Proc. Natl. Acad. Sci. U.S.A., 92:9146-50 (1995); Kinsella et al., Human Gene Therapy, 7:1405-13; Hofmann et al.,Proc. Natl. Acad. Sci. U.S.A., 93:5185-90; Choate et al., Human Gene Therapy, 7:2247 (1996); PCT/US97/01019 and PCT/US97/01048, and references cited therein, all of which are hereby expressly incorporated by reference.

The Apop proteins of the present invention are produced by culturing a host cell transformed with nucleic acid, preferably an expression vector, containing nucleic acid encoding an Apop protein, under the appropriate conditions to induce or cause expression of the Apop protein. The conditions appropriate for Apop protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

Appropriate host cells include yeast, bacteria, archaebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, SF9 cells, C129 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, fibroblasts, Schwanoma cell lines, immortalized mammalian myeloid and lymphoid cell lines, Jurkat cells, mast cells and other endocrine and exocrine cells, and neuronal cells. See the ATCC cell line catalog, hereby expressly incorporated by reference.

In a preferred embodiment, the Apop proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for Apop3 protein into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The

TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenlytion signals include those derived form SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

In a preferred embodiment, Apop proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of Apop3 protein into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.

In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E. coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

The expression vector may also include a signal peptide sequence that provides for secretion of the Apop protein in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria).

The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis, E. coli, Streptococcus cremoris*, and *Streptococcus lividans*, among others.

The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

In one embodiment, Apop proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art and are described e.g., in O'Reilly et al., *Baculovirus Expression Vectors: A Laboratory Manual* (New York: Oxford University Press, 1994).

In a preferred embodiment, Apop protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillerimondii* and *P. pastoris*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol

dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

The Apop protein may also be made as a fusion protein, using techniques well known in the art. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the Apop protein may be fused to a carrier protein to form an immunogen. Alternatively, the Apop protein may be made as a fusion protein to increase expression, or for other reasons. For example, when the Apop protein is an Apop peptide, the nucleic acid encoding the peptide may be linked to other nucleic acid for expression purposes.

In one embodiment, the Apop nucleic acids, proteins and antibodies of the invention are labeled. By "labeled" herein is meant that nucleic acids, proteins and antibodies of the invention have at least one element, isotope or chemical compound attached to enable the detection of nucleic acids, proteins and antibodies of the invention. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the compound at any position.

In a preferred embodiment, the Apop protein is purified or isolated after expression. Apop proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the Apop protein may be purified using a standard anti-Apop3 antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the Apop3 protein. In some instances no purification will be necessary.

Once expressed and purified if necessary, the Apop proteins and nucleic acids are useful in a number of applications.

In a preferred embodiment, the Apop proteins are used to make Apop antibodies. Apop antibodies find use in a number of applications. For example, the Apop antibodies may be coupled to standard affinity

chromatography columns and used to purify Apop proteins. The antibodies may also be used as blocking polypeptides, as outlined above, since they will specifically bind to the Apop protein.

In a preferred embodiment, the Apop protein and/or Apop nucleic acid is used to generate antibodies, for example for immunotherapy. By "immunotherapy" is meant treatment of apoptosis with an antibody raised against Apop proteins. As used herein, immunotherapy can be passive or active. Passive immunotherapy, as defined herein, is the passive transfer of antibody to a recipient (patient). Active immunization is the induction of antibody and/or T-cell responses in a recipient (patient). Induction of an immune response can be the consequence of providing the recipient with an Apop antigen to which antibodies are raised. As appreciated by one of ordinary skill in the art, the Apop antigen may be provided by injecting an Apop polypeptide against which antibodies are desired to be raised into a recipient, or contacting the recipient with an Apop nucleic acid, capable of expressing the Apop antigen, under conditions for expression of the Apop antigen.

In a preferred embodiment, a therapeutic compound is conjugated to an antibody, preferably an Apop antibody. The therapeutic compound may be a cytotoxic agent. In this method, targeting the cytotoxic agent to apoptotic cells or tumor tissue or cells, results in a reduction in the number of afflicted cells, thereby reducing symptoms associated with apoptosis and cancer. Cytotoxic agents are numerous and varied and include, but are not limited to, cytotoxic drugs or toxins or active fragments of such toxins. Suitable toxins and their corresponding fragments include diptheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin and the like. Cytotoxic agents also include radiochemicals made by conjugating radioisotopes to antibodies raised against Apop proteins, or binding of a radionuclide to a chelating agent that has been covalently attached to the antibody.

In a preferred embodiment, Apop genes are administered as DNA vaccines, either single genes or combinations of Apop genes. Naked DNA vaccines are generally known in the art; see Brower, Nature Biotechnology 16:1304-1305 (1998). Methods for the use of genes as DNA vaccines are well known to one of ordinary skill in the art, and include placing an Apop gene or portion of an Apop gene under the control of a promoter for expression in a patient. The Apop gene used for DNA vaccines can encode full-length Apop proteins, but more preferably encodes portions of the Apop proteins including peptides derived from the Apop protein. In a preferred embodiment a patient is immunized with a DNA vaccine comprising a plurality of nucleotide sequences derived from a Apop gene. Similarly, it is possible to immunize a patient with a plurality of Apop genes or portions thereof, as defined herein. Without being bound by theory, following expression of the polypeptide encoded by the DNA vaccine, cytotoxic T-cells, helper T-cells and antibodies are induced which recognize and destroy or eliminate cells expressing Apop proteins.

In a preferred embodiment, the DNA vaccines include a gene encoding an adjuvant molecule with the DNA vaccine. Such adjuvant molecules include cytokines that increase the immunogenic response to the Apop polypeptide encoded by the DNA vaccine. Additional or alternative adjuvants are known to those of ordinary skill in the art and find use in the invention.

In a preferred embodiment, the Apop proteins, nucleic acids, modified proteins and cells containing the native or modified Apop proteins are used in screening assays. Identification of the Apop proteins permits the design of drug screening assays for compounds that modulate Apop activity.

Screens are designed to first find candidate agents that can bind to Apop proteins, and then these agents are used in assays that evaluate the ability of the candidate agent to modulate Apop activity. The terms "binding to Apop" and "modulating Apop activity" or grammatical equivalents thereof comprise binding to Apop1 proteins, Apop2 proteins, Apop3 proteins, and modulating Apop1 protein activity, Apop2 protein activity, and Apop3 protein activity. As will be appreciated by those in the art, there are a number of different assays which may be run; binding assays and activity assays.

In a preferred embodiment, the methods comprise combining an Apop protein and a candidate bioactive agent, and determining the binding of the candidate agent to the Apop protein. Preferred embodiments utilize the human Apop protein, although other mammalian Apop proteins may also be used, including rodents (mice, rats, hamsters, guinea pigs, etc.), farm animals (cows, sheep, pigs, horses, etc.) and primates. These latter embodiments may be preferred in the development of animal models of human disease. In some embodiments, as outlined herein, variant or derivative Apop proteins may be used, including deletion Apop proteins as outlined above. Furthermore, included within the definition of Apop proteins are portions or fragments of Apop proteins; that is, either the full-length protein may be used, or, as outlined above, portions or fragments thereof. In addition, the assays described herein may utilize either isolated Apop proteins or cells comprising the Apop proteins.

Generally, in a preferred embodiment of the methods herein, the Apop protein or the candidate agent, is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g. a microtiter plate, an array, etc.). The insoluble support may be made of any composition to which the Apop protein or the candidate agent can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, teflon™, etc. Microtiter plates and arrays are especially

convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding the Apop protein or the candidate agent is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the Apop protein or candidate agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

In a preferred embodiment, the Apop protein is bound to the support, and a candidate bioactive agent is added to the assay. Alternatively, the candidate agent is bound to the support and the Apop protein is added. Novel binding agents include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for agents that have a low toxicity for human cells. Determination of the binding of an Apop protein and a candidate agent is done using a wide variety of assays, including, but not limited to labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

The term "candidate bioactive agent" or "exogeneous compound" as used herein includes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, lipid, etc., or mixtures thereof, with the capability of directly or indirectly altering the bioactivity of Apop. Generally a plurality of assay mixtures is run in parallel with different candidate agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids,

purines, pyrimidines, derivatives, structural analogs or combinations thereof. Particularly preferred are peptides.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

In a preferred embodiment, the candidate bioactive agents are proteins. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations.

In a preferred embodiment, the candidate bioactive agents are naturally occurring proteins or fragments of naturally occurring proteins. Thus, for example, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of procaryotic and eucaryotic proteins may be made for screening against Apop. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred.

In a preferred embodiment, the candidate bioactive agents are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below)

are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

In a preferred embodiment a library of protein encoding nucleotide sequences is added to the host cell comprising the vector composition of the invention. The library of protein encoding nucleotide sequences may be obtained from genomic DNA, from cDNAs or from random nucleotides. Particularly preferred in this embodiment are libraries encoding bacterial, fungal, viral, and mammalian proteins and peptides, with the latter being preferred, and human encoding proteins and peptides being especially preferred. As described above and as known in the art the protein and peptide encoding nucleotide sequences may be inserted into any vector suitable for expression in mammalian cells.

In a preferred embodiment, the candidate bioactive agents are nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference).

Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to increase the stability and half-life of such molecules in physiological environments. In addition, mixtures of naturally occurring nucleic acids and analogs can be made. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made. The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

As described above generally for proteins, nucleic acid candidate bioactive agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, the candidate bioactive agents are obtained from combinatorial chemical libraries, a wide variety of which are available in the literature. By "combinatorial chemical library" herein is meant a collection of diverse chemical compounds generated in a defined or random manner, generally by chemical synthesis. Millions of chemical compounds can be synthesized through combinatorial mixing.

The determination of the binding of the candidate bioactive agent to the Apop protein may be done in a number of ways. In a preferred embodiment, the candidate bioactive agent is labeled, and binding determined directly. For example, this may be done by attaching all or a portion of the Apop protein to

a solid support, adding a labeled candidate agent (for example a candidate agent comprising a fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as is known in the art.

By "labeled" herein is meant that the candidate bioactive agent is either directly or indirectly labeled with a label which provides a detectable signal, e.g. a radioisotope (such as ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, or ^{125}I), a fluorescent or chemiluminescent compound (such as fluorescein isothiocyanate, rhodamine, or luciferin), an enzyme (such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase), antibodies, particles such as magnetic particles, or specific binding molecules, etc. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule which provides for detection, in accordance with known procedures, as outlined above. The label can directly or indirectly provide a detectable signal.

In some embodiments, only one of the components is labeled. For example, the proteins (or proteinaceous candidate agents) may be labeled at tyrosine positions using ^{125}I, or with fluorophores. Alternatively, more than one component may be labeled with different labels; using ^{125}I for the proteins, for example, and a fluorophor for the candidate agents.

In a preferred embodiment, the binding of the candidate bioactive agent is determined through the use of competitive binding assays. In this embodiment, the competitor is a binding moiety known to bind to the target molecule (i.e. the Apop protein), such as an antibody, peptide, binding partner, ligand, etc. In a preferred embodiment, the competitor for Apop3 is RIP (Stanger et al., supra). In another preferred embodiment, the competitor for Apop1 and Apop2 is XIAP (Deveraux et al., Nature 388:300-303 (1997). Under certain circumstances, there may be competitive binding as between the bioactive agent and the binding moiety, with the binding moiety displacing the bioactive agent.

In one embodiment, the candidate bioactive agent is labeled. Either the candidate bioactive agent, or the competitor, or both, is added first to the protein for a time sufficient to allow binding, if present. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4°C and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high through put screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

In a preferred embodiment, the competitor is added first, followed by the candidate bioactive agent. Displacement of the competitor is an indication that the candidate bioactive agent is binding to the Apop protein and thus is capable of binding to, and potentially modulating, the activity of the Apop protein. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the candidate bioactive agent is labeled, the presence of the label on the support indicates displacement.

In an alternative embodiment, the candidate bioactive agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the bioactive agent is bound to the Apop protein with a higher affinity. Thus, if the candidate bioactive agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the candidate agent is capable of binding to the Apop protein.

In a preferred embodiment, the methods comprise differential screening to identity bioactive agents that are capable of modulating the activity of the Apop proteins. In this embodiment, the methods comprise combining an Apop protein and a competitor in a first sample. A second sample comprises a candidate bioactive agent, an Apop protein and a competitor. Addition of the candidate bioactive agent is performed under conditions which allow the modulation of the Apop protein. The binding of the competitor is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to the Apop protein and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to the Apop protein.

Another preferred embodiment utilizes differential screening to identify drug candidates that bind to the native Apop protein, but cannot bind to modified Apop proteins. The structure of the Apop protein may be modeled, and used in rational drug design to synthesize agents that interact with that site. Drug candidates that affect Apop bioactivity are also identified by screening drugs for the ability to either enhance or reduce the activity of the protein.

Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding.

Screening for agents that modulate the activity of Apop may also be done. In a preferred embodiment, methods for screening for a bioactive agent capable of modulating the activity of Apop comprise the steps of adding a candidate bioactive agent to a sample of Apop, as above, and determining an alteration in the biological activity of Apop. "Modulating the activity of Apop" includes an increase in activity, a decrease in activity, or a change in the type or kind of activity present. Thus, in this embodiment, the candidate agent should both bind to Apop (although this may not be necessary), and alter its biological or biochemical activity as defined herein. The methods include both in vitro screening methods, as are generally outlined above, and in vivo screening of cells for alterations in the presence, distribution, activity or amount of Apop.

Thus, in this embodiment, the methods comprise combining an Apop sample and a candidate bioactive agent, and evaluating the effect on apoptosis. By "Apop activity" or grammatical equivalents herein is meant one or more of the biological activities of Apop1, Apop2, and Apop3. Apop1's and Apop2's biological activity, e.g., comprises the ability to affect apoptosis. Apop3's biological activities, include, but are not limited to, its kinase activity, its ability to activate NF-κB, its ability to activate caspase-3, and its ability to affect apoptosis. Apop3 kinase activity may be assayed using known serine/threonine kinase assays; see Thome et al., J. Exp. Med 181:1997 (1995); del Peso et al., Science 278:687 (1997), and others. Similarly, death domain assays are known in the art.

In a preferred embodiment, the activity of the Apop protein is increased; in another preferred embodiment, the activity of the Apop protein is decreased. Thus, bioactive agents that are antagonists are preferred in some embodiments, and bioactive agents that are agonists may be preferred in other embodiments.

In a preferred embodiment, the invention provides methods for screening for bioactive agents capable of modulating the activity of an Apop protein. The methods comprise adding a candidate bioactive agent, as defined above, to a cell comprising Apop proteins. Preferred cell types include almost any cell as defined above for host cells. Preferred cells include, but are not limited to prokaryotic cells and eukaryotic cells, with mammalian cells and particularly human cells being preferred.

The cells contain a recombinant nucleic acid that encodes an Apop protein. In a preferred embodiment, a library of candidate agents is tested on a plurality of cells. By a "plurality of cells" herein is meant roughly from about 10^3 cells to 10^8 or 10^9, with from 10^6 to 10^8 being preferred.

In some embodiments, the assays include exposing the cells to an apoptosis agent that will induce apoptosis in control cells, i.e. cells of the same type but that do not contain the exogeneous nucleic acid encoding an Apop. Suitable apoptosis inducing agents are known in the art. Alternatively, the cells may be exposed to conditions that normally result in apoptosis, and changes in the normal apoptosis progression are determined. Alternatively, the cells into which the Apop nucleic acids are introduced, normally undergo apoptosis, and thus changes (for example, inhibition of apoptosis) are determined. Optionally, the cells normally do not undergo apoptosis, and the introduction of a candidate agent causes apoptosis.

Thus, the effect of the candidate agent on apoptosis is then evaluated.

Methods for the detection of apoptosis may be done as will be appreciated by those in the art. In one embodiment, indicators of apoptosis are used. Suitable apoptosis labels include, but are not limited to, DAPI. Accordingly, these agents can be used as an affinity ligand, and attached to a solid support such as a bead, a surface, etc. and used to pull out cells that are undergoing apoptosis. Similarly, these agents can be coupled to a fluorescent dye such as PerCP, and then used as the basis of a fluorescent-activated cell sorting (FACS) separation.

Sensitive assays that measure various biological and morphological hallmarks of the apoptotic process are known in the art. For example, a monoclonal antibody which can be used to detect cleavage of poly (ADP-ribose) polymerase, CCP32/Caspase-3 fluorescent and colorimetric assay kits, and a FLICE/caspase-8 fluorescent assay kit are commercially available (Clontech Laboratories, Inc.). Annexin V apoptosis assays, measuring the translocated phosphatidylserine (PS) are described, e.g., in Dachary-Prigent et al., Blood 81:2554-65 (1993); Thiagarajan and Tait, J. Biol. Chem. 265:17420-3 (1990); and Zhang et al., Biotechniques 23:525-31 (1997). Identification of apoptosis in situ via specific labeling of nuclear DNA fragmentation is described in Gavrieli et al., J. Cell Biol. 119:493-501 (1992).

In a preferred embodiment, bioactive agents identified by one of the methods outlined above, may be compounds showing pharmacological activity or therapeutical activity. Compounds with pharmacological activity are able to enhance or interfere with the activity of the Apop protein. The

compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host, as previously described. The agents may be administered in a variety of ways, orally, parenterally e.g., subcutaneously, intraperitoneally, intravascularly, etc. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of a therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

Without being bound by theory, it appears that the Apop proteins are important proteins in apoptosis. Accordingly, disorders based on mutant or variant Apop genes may be determined. In one embodiment, the invention provides methods for identifying cells containing variant Apop genes comprising determining all or part of the sequence of at least one endogenous Apop genes in a cell. As will be appreciated by those in the art, this may be done using any number of sequencing techniques. In a preferred embodiment, the invention provides methods of identifying the Apop genotype of an individual comprising determining all or part of the sequence of at least one Apop gene of the individual. This is generally done in at least one tissue of the individual, and may include the evaluation of a number of tissues or different samples of the same tissue. The method may include comparing the sequence of the sequenced Apop gene to a known Apop gene, i.e. a wild-type gene (e.g., those nucleic acid sequences depicted in Figures 1, 3, and 5) and determining if any differences exist. This can be done using any number of known homology programs, such as Bestfit, etc. In a preferred embodiment, the presence of a difference in the sequence between the Apop gene of the patient and the known Apop gene is indicative of a disease state or a propensity for a disease state, as outlined herein.

The present discovery relating to the role of Apop in apoptosis thus provides methods for inducing or preventing apoptosis in cells. In a preferred embodiment, the Apop proteins, and particularly Apop fragments, are useful in the study or treatment of conditions which are mediated by apoptosis, i.e. to diagnose, treat or prevent apoptosis-mediated disorders. Thus, "apoptosis mediated disorders" or "disease state" include conditions involving both insufficient apoptosis, including cancer, autoimmune

disorders and sustained viral infections, and excessive apoptosis, including inappropriate cell loss and degenerative disorders.

In one embodiment, methods of modulating apoptosis in cells or organisms are provided. In one embodiment, the methods comprise administering to a cell an anti-Apop antibody that reduces or eliminates the biological activity of the endogenous Apop protein. Alternatively, the methods comprise administering to a cell or organism a recombinant nucleic acid encoding an Apop protein. As will be appreciated by those in the art, this may be accomplished in any number of ways. In a preferred embodiment, the activity of Apop is increased by increasing the amount of Apop in the cell, for example by overexpressing the endogenous Apop or by administering a gene encoding Apop, using known gene-therapy techniques, for example. In a preferred embodiment, the gene therapy techniques include the incorporation of the exogeneous gene using enhanced homologous recombination (EHR), for example as described in PCT/US93/03868, hereby incorporated by reference in its entirety.

In one embodiment, the invention provides methods for diagnosing an Apop mediated disorder in an individual. The methods comprise measuring the activity and expression of Apop in a tissue from an individual or patient, which may include a measurement of the amount or specific activity of Apop. This activity is quantified and compared to the activity of Apop from either an unaffected second individual or from an unaffected tissue from the first individual. When these activities are different, the first individual may be at risk for an Apop mediated disorder. In this way, for example, monitoring of various disease conditions may be done, by monitoring the levels of Apop. Similarly, Apop levels may correlate to the diseases and conditions enumerated above.

In one aspect, the expression levels of Apop genes are determined in different patient samples or cells for which either diagnosis or prognosis information is desired. Gene expression monitoring is done on genes encoding Apop proteins. In one aspect, the expression levels of Apop genes are determined for different cellular states, such as normal cells and cells undergoing apoptosis. By comparing Apop gene expression levels in cells in different states, information including both up- and down-regulation of Apop genes is obtained, which can be used in a number of ways. For example, the evaluation of a particular treatment regime may be evaluated: does a chemotherapeutic drug act to improve the long-term prognosis in a particular patient. Similarly, diagnosis may be done or confirmed by comparing patient samples. Furthermore, these gene expression levels allow screening of drug candidates with an eye to mimicking or altering a particular expression level. This may be done by making biochips comprising sets of important Apop genes, such as those of the present invention, which can then be used in these screens. These methods can also be done on the protein basis; that is, protein

expression levels of the Apop proteins can be evaluated for diagnostic purposes or to screen candidate agents. In addition, the Apop nucleic acid sequences can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or the Apop proteins administered as therapeutic drugs.

Apop sequences bound to biochips include both nucleic acid and amino acid sequences as defined above. In a preferred embodiment, nucleic acid probes to Apop nucleic acids (both the nucleic acid sequences having the sequences outlined in Figures 1, 3, and 5 and/or the complements thereof) are made. The nucleic acid probes attached to the biochip are designed to be substantially complementary to the Apop nucleic acids, i.e. the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.

A "nucleic acid probe" is generally single stranded but can be partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. In general, the nucleic acid probes range from about 8 to about 100 bases long, with from about 10 to about 80 bases being preferred, and from about 30 to about 50 bases being particularly preferred. In some embodiments, much longer nucleic acids can be used, up to hundreds of bases (e.g., whole genes).

As will be appreciated by those in the art, nucleic acids can be attached or immobilized to a solid support in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one or more of either electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at

least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

The biochip comprises a suitable solid substrate. By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not appreciably show fluorescence.

In a preferred embodiment, the surface of the biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. Thus, for example, the biochip is derivatized with a chemical functional group including, but not limited to, amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. Using these functional groups, the probes can be attached using functional groups on the probes. For example, nucleic acids containing amino groups can be attached to surfaces comprising amino groups, for example using linkers as are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). In addition, in some cases, additional linkers, such as alkyl groups (including substituted and heteroalkyl groups) may be used.

In this embodiment, oligonucleotides, corresponding to the nucleic acid probe, are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside.

In an additional embodiment, the immobilization to the solid support may be very strong, yet non-covalent. For example, biotinylated oligonucleotides can be made, which bind to surfaces covalently coated with streptavidin, resulting in attachment.

Alternatively, the oligonucleotides may be synthesized on the surface, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques are used. In a preferred embodiment, the nucleic acids can be synthesized in situ, using well known photolithographic techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Patent Nos. 5,700,637 and 5,445,934; and references cited within, all of which are expressly incorporated by reference; these methods of attachment form the basis of the Affimetrix GeneChip™ technology.

"Differential expression," or grammatical equivalents as used herein, refers to both qualitative as well as quantitative differences in the genes' temporal and/or cellular expression patterns within and among the cells. Thus, a differentially expressed gene can qualitatively have its expression altered, including an activation or inactivation, in, for example, normal versus apoptotic cell. That is, genes may be turned on or turned off in a particular state, relative to another state. As is apparent to the skilled artisan, any comparison of two or more states can be made. Such a qualitatively regulated gene will exhibit an expression pattern within a state or cell type which is detectable by standard techniques in one such state or cell type, but is not detectable in both. Alternatively, the determination is quantitative in that expression is increased or decreased; that is, the expression of the gene is either upregulated, resulting in an increased amount of transcript, or downregulated, resulting in a decreased amount of transcript. The degree to which expression differs need only be large enough to quantify via standard characterization techniques as outlined below, such as by use of Affymetrix GeneChip™ expression arrays, Lockhart, Nature Biotechnology 14:1675-1680 (1996), hereby expressly incorporated by reference. Other techniques include, but are not limited to, quantitative reverse transcriptase PCR, Northern analysis and RNase protection.

As will be appreciated by those in the art, this may be done by evaluation at either the gene transcript, or the protein level; that is, the amount of gene expression may be monitored using nucleic acid probes to the DNA or RNA equivalent of the gene transcript, and the quantification of gene expression levels, or, alternatively, the final gene product itself (protein) can be monitored, for example through the use of antibodies to the Apop protein and standard immunoassays (ELISAs, etc.) or other techniques, including mass spectroscopy assays, 2D gel electrophoresis assays, etc.

In another method detection of the mRNA is performed in situ. In this method permeabilized cells or tissue samples are contacted with a detectably labeled nucleic acid probe for sufficient time to allow

the probe to hybridize with the target mRNA. Following washing to remove the non-specifically bound probe, the label is detected. For example a digoxygenin labeled riboprobe (RNA probe) that is complementary to the mRNA encoding an Apop protein is detected by binding the digoxygenin with an anti-digoxygenin secondary antibody and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

In another preferred method, expression of Apop proteins is performed using in situ imaging techniques employing antibodies to Apop proteins. In this method cells are contacted with from one to many antibodies to the Apop protein(s). Following washing to remove non-specific antibody binding, the presence of the antibody or antibodies is detected. In one embodiment the antibody is detected by incubating with a secondary antibody that contains a detectable label. In another method the primary antibody to the Apop protein(s) contains a detectable label. In another preferred embodiment each one of multiple primary antibodies contains a distinct and detectable label. This method finds particular use in simultaneous screening for a pluralilty of Apop proteins. The label may be detected in a fluorometer which has the ability to detect and distinguish emissions of different wavelengths. In addition, a fluorescence activated cell sorter (FACS) can be used in this method. As will be appreciated by one of ordinary skill in the art, numerous other histological imaging techniques are useful in the invention and the antibodies can be used in ELISA, immunoblotting (Western blotting), immunoprecipitation, BIACORE technology, and the like.

In one embodiment, a therapeutically effective dose of an Apop is administered to a patient. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for Apop degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and organisms. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

The administration of the Apop proteins of the present invention can be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally,

intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly. In some instances, for example, in the treatment of wounds and inflammation, the Apop may be directly applied as a solution or spray.

The pharmaceutical compositions of the present invention comprise an Apop protein in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. Additives are well known in the art, and are used in a variety of formulations.

In another preferred embodiment, antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of Apop genes *in vivo*. The term "antisense" herein means that the nucleic acid sequence of the antisense RNA or DNA comprises the reverse complement sequence of the mRNA to which it can bind. For example, if the mRNA, whose expression (i.e., translation into a protein) is to be blocked, comprises the nucleic acid sequence 5'-GGAAUUGGAGC-3', then the antisense RNA comprises the nucleic acid sequence of 5'-GCUCCAAUUCC-3' and the antisense DNA comprises the nucleic acid sequence of 5'-GCTCCAATTCC-3'.

An Apop antisense molecule as defined herein is a nucleic acid molecule which exhibits at least 75%, more preferably at least 80%, even more preferably at least 90%, and most preferably more than at least 95% reverse-complement identity to its target sequence. In one embodiment herein, an Apop antisense molecule exhibits 100% reverse-complement identity to its target sequence. As known in the art, the antisense nucleic acid can comprise nucleotide homologues, such as inosine, etc.

An Apop antisense molecule as defined herein is a nucleic acid molecule which inhibits expression or translation of an Apop nucleic acid by at least 30%, preferably by at least 40%, more preferably by at least 50%, even more preferably by at least 70%, and most preferably by at least 90%. In one embodiment herein, an Apop antisense molecule inhibits expression or translation of mRNA encoding Apop 100%.

Generally, the Apop antisense molecule is at least about 10 nucleotides in length, more preferably at least 12, and most preferably at least 15 nucleotides in length. The skilled artisan understands that the length can extend from 10 nucleotides or more to any length which does not inhibit binding to the Apop nucleic acid. In a preferred embodiment herein, the length is about 100 nucleotides long, more preferably about 50 nucleotides, more preferably about 25 nucleotides, and most preferably about 12 to 25 nucleotides in length.

It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane [Zamecnik et al., Proc. Natl. Acad. Sci. U.S.A. 83:4143-4146 (1986)]. The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection [Dzau et al., Trends in Biotechnology 11:205-210 (1993)]. In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target

intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., J. Biol. Chem. 262:4429-4432 (1987); and Wagner et al., Proc. Natl. Acad. Sci. U.S,A. 87:3410-3414 (1990). For review of gene marking and gene therapy protocols see Anderson et al., Science 256:808-813 (1992).

In a preferred embodiment, the Apop proteins of the present inventions are used as "bait proteins" in a yeast two-hybrid screening or in a mammalian two-hybrid screening (Luo et al., Biotechniques 22:350-352 (1997) and US application SN 09/050,863, both of which are expressly incorporated as references in their entirety) to isolate novel proteins interacting with the Apop proteins.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

EXAMPLE 1

Isolation and sequence analysis of Apop3

Several known TNFα signaling proteins have been shown to bind RIP [Hsu et al., Immunity 4:387-396 (1996)] and complexes formed by the interactions between RIP kinase, TRADD, FADD, and RAIDD recruit other proteins to TNFR1 or Fas receptors to initiate signaling [Stanger et al., Cell 81:513-523 (1995); Hsu et al., Immunity 4:387-396 (1996); Tartaglia et al., Cell 74:845-853 (1993); Darnay et al., J. Leukoc. Biol. 61:559-566 (1997); Ashkenazi and Dixit, Science 281:1305-1308 (1998)]. We established a large-scale yeast two hybrid screening system designed to isolate rare mRNAs and verify novel protein-protein interactions involved in the TNF signaling pathway. Using RIP as bait, we screened 96 million independent yeast transformants with a combined Hela/Lymphocyte cDNA library in a single transformation round. As expected, many of the known RIP binding proteins, including TRAF1, TRAF2, TNFR1, RIP, TRADD, and FADD, were cloned; several novel genes were isolated as well. One of the novel cDNAs had high sequence homology with RIP. The full-length cDNA (Figure 5) contains a 518 amino acid open reading frame, encoding a protein designated Apop3. Analysis of the amino acid sequence of this novel protein (Figure 6) revealed a kinase domain (aa 21-287) which had 47% homology to the kinase domain of RIP and 42% homology to that of RIP2/Rick/CARDIAK. However, outside the kinase domain, the C-terminal region of Apop3 had no homology to death domains [Tartaglia et al., Cell 74:845-853 (1993)], death effector domains [Siegel et al., J. Cell. Biol. 141:1243-1253 (1998)], or the CARD region [Hofmann et al., Trends Biochem. Sci. 22:155-156 (1997)]. An approximately 2.1

Kb transcript was detected with a cDNA probe specific to Apop3 in both normal human tissue and human tumor RNA blots (data not shown).

EXAMPLE 2

Co-immunoprecipitation with Apop3 and other members of TNFα signaling complex

We confirmed the association of Apop3 and other members of TNFα signaling complex in mammalian cells by co-immunoprecipitation. To further analyze which region of Apop3 was important for interactions with RIP, a series of deletion mutants were engineered (Figure 7). In yeast, the intermediate region (aa 82–436) of Apop3 was required for the interaction between RIP and Apop3 (Figure 8). Co-immunoprecipitation analysis was performed to verify the interaction of RIP with Apop3 by co-transfection of HA-tagged RIP with the Flag-tagged full length and truncated mutants of Apop3 in Phoenix-A (293 T) cells. In this example, cell lysates were collected from Phoenix-A cells 24 hours after the co-transfection of HA-RIP (5 μg) with a Flag-control protein or with one of the Flag-Apop3 proteins, schematically depicted in Figure 7 (5 μg). Co-precipitated Flag-Apop3 proteins were detected by immunoblotting with anti-Flag polyclonal Ab. The full length Apop3 co-immunoprecipitated with RIP, as well as Apop3(82-518) and Apop3(K50D) mutants (data not shown). Interestingly, the Apop3(1-436) mutant has dramatically reduced binding affinity for RIP. No association was detected between RIP and Apop3(1-251), Apop3(1-196), or Apop3(287-518). This suggests that the interaction between Apop3 and RIP is mediated by a portion of the kinase domain with contribution from the carboxyl-domain of Apop3.

EXAMPLE 3

Apop3 binds to endogenous RIP

To test whether Apop3 was able to bind to endogenous RIP, Flag-Apop3 was transfected into Phoenix-A (293 T) cells in the presence and absence of TNFα stimulation. In this example, 293 T cells were transfected with Flag-control protein, and Flag-Apop3 (10 μg). After 18 hours incubation, cells were treated with or without 20 ng/ml huTNFα for 6 hours. The cell extracts were harvested 24 hours after transfection. The immunoprecipitated proteins were immunoblotted with goat anti-RIP polyclonal Ab. Ectopically expressed Apop3 was able to bind to endogenous RIP kinase (data not shown). TNFα treatment did not significantly affect this binding (data not shown).

EXAMPLE 4

Endogenous Apop3 binds to TRAF2 and TNFR1

In addition, endogenous TNFR1 was co-immunoprecipitated with ectopically expressed Apop3 and endogenous Apop3 was co-immunoprecipitated with ectopically expressed TRAF2 (data not shown). Interaction between TRAF2 and Apop3 was also verified by the yeast two-hybrid system (data not shown). This suggests that Apop3 is part of the TNFR1 signaling complex. In this example, Flag-tagged TRAF2 expression vector was transfected into cells. Association with endogenous Apop3 was detected by anti-Apop3 Ab. Further, Flag-tagged Apop3 was transfected into cells ans association with endogenous TNFR1 was detected by anti-TNFR1 Ab (data not shown)

EXAMPLE 5

Demonstration of APOP3 kinase activity

Apop3 contains consensus Ser/Thr kinase motifs. The lysine 50 of the Apop3 kinase domain, homologous to the conserved lysine in RIP and critical for enzymatic activity and ATP-binding [McCarthy et al., J. Biol. Chem. 273:16968-16975 (1998); Inohara et al., J. Bio. Chem. 273: 12296-12300 (1998)], was substituted with aspartic acid. An *in vitro* kinase assay demonstrated that Apop3 was a kinase and was autophosphorylated (data not shown). However, the Apop3(K50D) mutant lost the kinase activity (data not shown), suggesting that this lysine is required for autophosphorylation. In this example, Phoenix-A cells were transfected with 10 µg of a Flag-vector, Flag-Apop3 and Flag-Apop3(K50D), respectively. The lysates were immunoblotted with anti-Flag mAb to adjust protein amount for IP.

EXAMPLE 6

Induction of cell death by Apop3

To test whether Apop3 was able to induce cell death, Phoenix-A cells were co-transfected with a eukaryotic expression vector encoding Apop3 and a pGDB control for transfection efficiency [Xu et al., Nucl. Acids Res. 26:2034-2035 (1998)]. The GFP-positive Apop3-transfected cells displayed morphological characteristics of apoptosis, including detachment from the plate substrate and resultant "rounding up" and a condensed/fragmented nucleus [Cryns et al., Cell 82:349-352 (1995)] (data not shown). The C-terminal deletion mutant, Apop3(1-436), failed to induce apoptosis. Apop3-induced apoptosis was also observed in Hela cells (data not shown). Figure 9 shows approximately 35% cell death in the Apop3 transfected Phoenix-A cells, compared with only 2% apoptosis in cells transfected with control vector. Similar to RIP [Hsu et al., Immunity 4:387-396 (1996)] and RIP2/Rick/CARDIAK kinase deletions [McCarthy et al., J. Biol. Chem. 273:16968-16975 (1998)], the deletion of the Apop3 kinase domain had no effect on its pro-apoptotic activity (data not shown), which suggested the kinase domain was not required for Apop3-mediated cell death.

EXAMPLE 7

Activation of cellular caspases by Apop3

TNFα and Fas ligand induced apoptosis is controlled by caspase activation [Martin and Green, Cell 82:349-352 (1995)]. Rick/RIP2/CARDIAK kinase has also been reported to induce apoptosis through enhancement of caspase activity [Inohara et al., J. Bio. Chem. 273: 12296-12300 (1998)]. To test whether Apop3 activates cellular caspases, pYCI-Apop3 (data not shown) was co-transfected into Phoenix-A cells with pGDB. In this example, 2 x 10^6 Phoenix-A cells were co-transfected with expression vectors encoding Apop3 and Apop3 mutants, such as depicted in Figure 7 (10 µg) with pGDB (3µg). Lysates were analyzed by Western blot probed with anti-GFP monoclonal antibodies. Caspase cleavage released the monomeric GFP as indicated by the arrow. pGDB expresses a previously described BFP-GFP hybrid protein linked by a DEVD containing peptide ('GFP'-GSGSGSDEVDGGSGSGS-'BFP', wherein the caspase cleavage site is in between V and D) used as a reporter of intracellular caspase activity [Xu et al., Nucl. Acids Res. 26:2034-2035 (1998)]. When caspases 2, 3, or 7 are activated, they specifically cleave appropriately accessible substrates at the DEVD peptide [Cryns et al., Cell 82:349-352 (1995)] and release GFP/BFP monomers. Western blot analysis using anti-GFP monoclonal antibody indicated that ectopic expression of Apop3 activated caspases to cleave the DEVD linker peptide, releasing GFP/BFP monomer (data not shown). A broad-spectrum inhibitor of caspases, zVAD-fmk [Zhu et al., FEBS Lett. 374:303-308 (1995)], inhibited the caspase activity triggered by Apop3 expression (data not shown). This result confirmed that the cleavage of the chimeric protein was due to caspase activation (data not shown). Apop3-induced apoptosis could also be inhibited by CrmA (data not shown). N-terminal deletion mutants Apop3(82-518), Apop3(287-518) and Apop3(K50D) were still able to activate the caspase activity (data not shown). Deletion of the C-terminal region of Apop3, Apop3(1-251) and Apop3(1-436), completely abrogated caspase activation (data not shown). These results indicate again that Apop3's kinase activity is not required for activation of caspases and cell death. Since the C-terminal domain (aa 437-518) of Apop3 is not required for the binding of Apop3 to RIP, this region may be involved in binding to other proteins involved in novel signaling pathway(s) leading to apoptosis. It is not known whether the C-terminal region of Apop3 is able to bind directly to known death adaptor or death protease. Additional two-hybrid screening using Apop3 as bait is being carried out to find signaling proteins downstream of Apop3.

EXAMPLE 8

Induction of activation of NFκB by Apop3

We also tested whether Apop3 could induce NFκB activation. Phoenix-A cells were harvested for a luciferase reporter assay 24 hours after co-transfection with an NFκB–dependent luciferase reporter construct and an Apop3 expression vector (pYCI-Apop3; data not shown). Ectopic expression of Apop3 alone induced NFκB activation approximately 7 fold (Figure 10 and data not shown). The NFκB activation

could be inhibited by co-transfection of a IκB dominant negative mutant (data not shown). The C-terminal region was essential for NFκB activation, as Apop3(1-251) and Apop3(1-436) were not able to activate NFκB (Figure 10). Interestingly, Apop3(K50D), Apop3(82-518), and Apop3(287-518) induced higher luciferase activity than full length Apop3 (Figure 10). This suggests that the kinase domain of Apop3 is not required for the NFκB activation. In contrast, Apop3 had no noticeable effect on AP-1 activation pathways (data not shown).

EXAMPLE 9

Overexpression of Apop3 inhibits TNFα-induced caspase activation but not TNFα-induced NFκB activation

Although Apop3 associates with RIP and TRAF2 and activates apoptosis and NFκB, its function in the TNFα signaling pathway needs to be dissected. In this example, Phoenix-A (293 T) cells were co-transfected with expression vectors encoding Apop3 and Apop3 mutants, such as depicted in Figure 7 (3μg) with pGDB (0.3 μg) and RIP (0.1 μg). After 24 hours of transfection, cell lysates were harvested and analyzed by Western blot analysis with anti-GFP monoclonal antibodies. In these experiments, Apop3(1-436) inhibited TNFα-induced caspase activation (data not shown) but not TNFα-induced NFκB activation (data not shown), suggesting that Apop3 is involved in the TNFα-mediated apoptosis pathway. These results imply that without the C-terminal domain (aa 437-518), Apop3 either competes with or fails to recruit other proteins of the TNF signaling of a complex required for the activation of caspases. In separate experiments, Apop3(1-436) was able to down-regulate RIP-mediated apoptosis activation (data not shown). Co-transfection of full length of Apop3 (3 μg) with RIP (0.1 μg) resulted in greater caspase activity than with RIP alone (data not shown). However, Apop3(1-436) significantly reduced RIP-induced caspase activity (data not shown). On the other hand, although Apop3 alone was able to activate NFκB, co-transfection of RIP (0.3 μg) and full length Apop3 (3 μg) in Phoenix cells did not show any synergistic effect on NFκB activation (data not shown). When Apop3(1-436) was co-transfected with RIP, it only slightly reduced RIP-mediated NFκB activation (data not shown). Another Apop3 mutant (aa 287-518) was still able to activate caspase and NFκB, even though it did not bind to RIP (data not shown).

In summary, we demonstrated that Apop3 is a novel kinase associated with the TNFα signaling complex. Apop3 binds to RIP and TRAF2 in both yeast and mammalian cells and co-immunoprecipitates with TNFR1. Although RIP is a essential player in TNFα induced NFκB activation [Kelliher et al,. Immunity 8:297-303 (1998)] and Apop3 was cloned by binding to RIP, the function of Apop3 may not rely solely on its binding to RIP. A dominant negative form of Apop3 is able to inhibit TNFα-induced caspase activation without affecting TNFα-induced NFκB activation, suggesting that Apop3 may be involved in TNFα-induced apoptosis. It is possible that Apop3 and RIP are therefore involved in two different

areas of TNFα signaling; apoptosis and NFκB activation, respectively. The precise role of Apop3 in TNFα signaling may be answered by future knock-out experiments.

EXAMPLE 10

The yeast two-hybrid screening method: Plasmids, cDNA libraries, yeast strains, growth media and retrieval of plasmids

Unknown proteins interacting with a known "Bait" protein can be identified using the yeast two-hybrid screening method. In this method, a known "Bait" protein (such as XIAP or RIP) is encoded on a "bait vector" and a protein (the "Test" protein) which may bind to the "Bait" protein is usually encoded on a "test" vector or "test" plasmid. The method comprises the following steps: (1) transforming yeast with a "bait" vector and a "test" vector (usually a library); (2) detecting an interaction between the bait protein and a test protein (usually by expression of a reporter gene such as lacZ); (3) growing the identified yeast colony; (4) isolating the "test" vector; (5) transforming the "test" vector into E.coli; and (6) using standard techniques to further characterize the "test" vector and the encoded "test' protein.

Plasmids and cDNA Libraries: The pAS2 and pACT2 plasmid series were originally constructed by Elledge and co-workers [Durfee et al., Genes Dev. 4:555-69 (1993)]. The bait vector used in our screening is either pAS2 or pAS2K. pAS2K was constructed by replacing the Amp^R gene in pAS2 with a Kan^R gene. cDNA libraries used in our screening were made, e.g., either from brain tissues or T and B lymphocytes.

The yeast two-hybrid system reporter strain Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4D, gal80D, cyhr2, LYS2::GAL1UAS-HIS3TATA-HIS3, URA3::GAL1UAS-GAL1TATA-lacZ) was used in all screeings.

All yeast culture mediums, including, e.g., YPD, YPD Agar, DOB, DOBA, CSM-TRP, CSM-LEU, CSM-HIS, CSM-URA, CSM-LYS, CSM-LEU-TRP, CSM-LEU-HIS, and CSM-LEU-TRP-HIS, are available from Bio101, Inc. 3AT (3-amino-1,2,4-triazol) is available from Sigma (Cat# A-8056, St. Louis, MO, USA).

There are several methods to retrieve plasmids from yeast, ranging from lyticase lysis to glass beads. The glass beads method is listed below:

1. Inoculate 3 ml of selection medium (e.g., SD-L for cDNA library plasmid pACT) with a yeast colony.
2. Incubate in a 30°C shaker or rotator overnight or until confluent.
3. Spin down yeast in a bench-top centrifuge at 3000 rpm at room temperature.

4. Remove medium and re-suspend pellet in 200 µl lysis buffer. Transfer to an eppendorf tube.
5. Add 200 µl volume glass beads.
6. Add 200 µl phenol/chloroform/isoamyl alcohol (25:24:1).
7. Vortex at the highest speed for 3 minutes.
8. Spin in micro-centrifuge at 14000 rpm for 10 minutes.
9. Transfer top water layer to another eppendor tube, add 20 µl 3M NaAc and 500 µl ethanol.
10. Put the eppendorf tube into a dry ice bath for 15 minutes or until frozen.
11. Spin in a micro-centrifuge 14000 rpm for 10 minutes.
12. Remove supernatant and dry pellet.
13. Wash pellet by 100 µl of 80% ethanol, and dry the pellet in air.
14. Re-suspend pellet in 30 µl H_2O and use 1µl for electroportion to transform *E. coli.*

Electroporation method is by far the most efficient method to transform plasmids from yeast miniprep into *E. coli.* Bait and cDNA (test) plasmids may carry different antibiotic selection markers to facilitate separation in *E. coli.* For example, our bait plasmid carries a Kan^R gene and the cDNA (test) plasmid carries an Amp^R gene.

CLAIMS

We claim:

1. A recombinant nucleic acid encoding an Apop3 protein that is at least about 85% identical to the amino acid sequence depicted in Figure 6.

2. A recombinant nucleic acid that is at least about 85% identical to the nucleic acid sequence depicted in Figure 5 or its complement.

3. A recombinant nucleic acid according to claim 1, wherein said Apop3 protein is a human Apop3 protein.

4. A recombinant nucleic acid according to claim 1 encoding the amino acid sequence depicted in Figure 6.

5. A recombinant nucleic acid according to claim 2 that will hybridize under high stringency conditions to the nucleic acid sequence depicted in Figure 5 or its complement.

6. A recombinant nucleic acid according to claim 1 operably linked to control sequences recognized by a host cell transformed with the nucleic acid.

7. An expression vector comprising the nucleic acid of any claims 1-6.

8. A host cell comprising the nucleic acid of any claims 1-7.

9. A process for producing an Apop3 protein comprising culturing the host cell of claim 8 under conditions suitable for expression of an Apop3 protein.

10. A process according to claim 9, further comprising recovering said Apop3 protein.

11. A recombinant Apop3 protein that is at least about 85% identical to the amino acid sequence depicted in Figure 6.

12. An Apop3 protein according to claim 11 comprising the sequence depicted in Figure 6.

13. An Apop3 protein according to claim 11 or 12 encoded by a nucleic acid that is at least about 85% identical to the nucleic acid sequence depicted in Figure 5.

14. An Apop3 protein according to claim 11, 12 or 13 encoded by a nucleic acid that will hybridize under high stringency conditions to the complement of the nucleic acid sequence depicted in Figure 5.

15. A monoclonal antibody which specifically binds to an Apop3 protein according to claim 11, 12, 13, or 14.

16. A monoclonal antibody according to claim 15 that reduces or eliminates the biological function of said Apop3 protein.

17. A method for screening for a bioactive agent capable of binding to an Apop3 protein, said method comprising:

a) combining an Apop3 protein and a candidate bioactive agent; and

b) determining the binding of said candidate bioactive agent to said Apop3 protein.

18. A method for screening for a bioactive agent capable of interfering with the binding of Apop3 and RIP, said method comprising:

a) combining an Apop3 protein, a candidate bioactive agent and a RIP protein; and

b) determining the binding of said Apop3 protein and said RIP protein.

19. A method for screening for a bioactive agent capable of modulating the activity of an Apop3 protein, said method comprising the steps of:

a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an Apop3 protein; and

b) determining the effect of the candidate bioactive agent on apoptosis.

20. A method according to claim 19, wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding an Apop3 protein.

```
 1    CACAGACAGGCTCTTAACTGTTTATATCACTCACTTGGGAAAGTGTCTCAAGCTGTTCTA
61    CAAATCCATGCAAAGGCCGTTTAAAAATAGCAGCGAAGGTCCTGGACTCGGTCTCGTCCA
121   GCACAGCCCCTTGGCTCTCTCTCTGGGCTCTGGCCGCCTGGCCCCCGGGGACCCACACGA
181   GGTCATGGCGTGCTTCGGGCAGGGGGGCGGGGATCCCATAGACACCTCAGCTCCTTAAGA
241   GTTCTCCGCCTGGGCCAGGACGAGCATGGGGGTCCCCACTGATGCCCGAGACAGTGCCCC
301   TGTGTGTGTGAGCCCTCGACCCACATAACAGAGAGGTGTCCTGATGCCCTCTGTCCTCTC
361   CAGGTGGATCTAGGATCCGGCTTCCAACATGTGGCAGCTCTGGGCCTCCCTCTGCTGCCT
421   GCTGGTGTTGGCCAATGCCCGGAGCAGGCCCTCTTTCCATCCCGTGTCGGATGAGCTGGT
481   CAACTATGTCAACAAACGGAATACCACGTGGCAGGCCGGGCACAACTTCTACAACGTGGA
541   CATGAGCTACTTGAAGAGGCTATGTGGTACCTTCCTGGGTGGGCCCAAGCCACCCCAGAG
601   AGTTATGTTTACCGAGGACCTGAAGCTGCCTGCAAGCTTCGATGCACGGGAACAATGGCC
661   ACAGTGTCCCACCATCAAAGAGATCAGAGACCAGGGCTCCTGTGGCTCCTGCTGGGTAAG
721   GCCCTGCTGGCTGGTGGGGAAGCGCTGGAGAGAAAGTGGGAGCAACACTGGAGAGTCTTG
781   GGGGATTCGGGGTGGGGACAACTCTGACAAGGCAAGTTATAGAAACTTTCTGAGTCCCAG
781   TTTCCATCAGTACAAAAATCACAATCCCTCTGGCCATGAATGAATGGCGAGGATTAGGTGG
841   AGTGGCGGGCAGAGCATCCAGCAGATTGCAAGTCCACGTGTACAGGTGGCGAAGCAGCTC
901   CCTTTCCCTGACATGCTGGCCCGTCCGCAAATACCAGGAGCTCTCACTGCTACTCTGCTT
961   CAAGAAAGCATCCCTTTAGTGTCAGTGAGCTGTCTTAATTTTGTCATTTAATTGTGGTAA
1021  AATACACGTAACAGAAATGTAATAATCTTAGCAATCTTCTTTTGTTTTCTTTTTCTTTTT
1081  TTTTTTTTTTTTTTT
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FIGURE 1

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  1 MWQLWASLCCLLVLANARSRPSFHPVSDELVNYVNKRNTTWQAGHNFYNVDMSYLKRLCG
 61 TFLGGPKPPQRVMFTEDLKLPASFDAREQWPQCPTIKEIRDQGSCGSCWVRPCWLVGKRW
121 RESGSNTGESWGIRGGDNSDKASYRNFLSPSFHQYKNHNPSGHE
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FIGURE 2

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   1 TCCAAGTCCTGGATGAGCTCGGAAGACACGTGCTGCTGCGGAAGGACTGTGGCCCAGTGG
  61 ACACCAAGGTTCCAGGTGCTGGGGAGCCCAAGTCAGCCTTCACTCAGGGCTCAGCCATGA
 121 TTTCTTCTCTGTCCCAAAACCATCCAGACAACAGAAACGAGACTTTCAGCTCAGTCATTT
 181 CCCTGTTGAATGAGGATCCCCTGTCCCAGGACTTGCCTGTGAAGATGGCTTCAATCTTCA
 241 AGAACTTTGTCATTACCTATAACCGGACATATGAGTCAAAGGAAGAAGCCCGGTGGCGCC
 301 TGTCCGTCTTTGTCAATAACATGGTGCGAGCACAGAAGATCCAGGCCCTGGACCGTGGCA
 361 CAGCTCAGTATGGAGTCACCAAGTTCAGTGATCTCACAGAGGAGGAGTTCCGCACTATCT
 421 ACCTGAATACTCTCCTGAGGAAAGAGCCTGGCAACAAGATGAAGCAAGCCAAGTCTGTGG
 481 GTGACCTCGCCCCACCTGAATGGGACTGGAGGAGTAAGGGGGCTGTCACAAAAGTCAAAG
 541 ACCAGGGCATGTGTGGCTCCTGCTGGGCCTTCTCAGTCACAGGCAATGTGGAGGGCCAGT
 601 GGTTTCTCAACCAGGGGACCCTGCTCTCCCTCTCTGAACAGGAGCTCTTGGACTGTGACA
 661 AGATGGACAAGGCCTGCATGGGCGGCTTGCCCTCCAATGCCTACTCGGCCATAAAGAATT
 721 TGGGAGGGCTGGAGACAGAGGATGACTACAGCTACCAGGGTCACATGCAGTCCTGCAACT
 781 TCTCAGCAGAGAAGGCCAAGGTCTACATCAATGACTCCGTGGAGCTGAGCCAGAACGAGC
 841 AGAAGCTGGCAGCCTGGCTGGCCAAGAGAGGCCCAATCTCCGTGGCCATCAATGCCTTTG
 901 GCATGCAGTTTTACCGCCACGGGATCTCCCGCCCTCTCCGGCCCCTCTGCAGCCCTTGGC
 961 TCATTGACCATGCGGTGTTGCTTGTGGGCTACGGCAACCGCTCTGACGTTCCCTTTTGGG
1021 CCATCAAGAACAGCTGGGGCACTGACTGGGGTGAGAAGGGTTACTACTACTTGCATCGCG
1081 GGTCCGGGGCCTGTGGCGTGAACACCATGGCCAGCTCGGCGGTGGTGGACTGAAGAGGGG
1141 CCCCCAGCTCGGGACCTGGTGCTGATCAGAGTGGCTGCTGCCCCAGCCTGACATGTGTCC
1201 AGGCCCCTCCCCGGGAGGTACAGCTGGCAGAGGGAAAGGCACTGGGTACCTCAGGGTGAG
1261 CAGAGGGCACTGGGCTGGGGCACAGCCCCTGCTT
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FIGURE 3

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1   MISSLSQNHPDNRNETFSSVISLLNEDPLSQDLPVKMASIFKNFVITYNRTYESKEEARW
61  RLSVFVNNMVRAQKIQALDRGTAQYGVTKFSDLTEEEFRTIYLNTLLRKEPGNKMKQAKS
121 VGDLAPPEWDWRSKGAVTKVKDQGMCGSCWAFSVTGNVEGQWFLNQGTLLSLSEQELLDC
181 DKMDKACMGGLPSNAYSAIKNLGGLETEDDYSYQGHMQSCNFSAEKAKVYINDSVELSQN
241 EQKLAAWLAKRGPISVAINAFGMQFYRHGISRPLRPLCSPWLIDHAVLLVGYGNRSDVPF
301 WAIKNSWGTDWGEKGYYYLHRGSGACGVNTMASSAVVD
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FIGURE 4

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   1 atgtcgtgcg tcaagttatg gcccagcggt gcccccgccc ccttggtgtc catcgaggaa
  61 ctggagaacc aggagctcgt cggcaaagac gggttcggca cagtgttccg ggcgcaacat
 121 aggaagtggg gctacgatgt ggcggtcaag atcgtaaact cgaaggcgat atccagggag
 181 gtcaaggcca tggcaagtct ggataacgaa ttcgtgttgc gcctagaagg ggttatcgag
 241 aaggtgaact gggaccaaga tcccaagccg gctctggtga ctaaattcat ggagaacggc
 301 tccctgtcgg ggctgctgca gtcccagtgc cctcggccct ggccgctcct ttgccgcctg
 361 ctgaaagaag tggtgcttgg gatgttttac ctgcacgacc agaacccggt gctcctgcac
 421 cgggacctca agccatccaa cgtcctgccg gacccagagc tgcacgtcaa gctggcagat
 481 tttggcctgt ccacatttca gggaggctca cagtcaggga cagggtccgg ggagccaggg
 541 ggcaccctgg gctacttggc cccagaactg tttgttaacg taaaccggaa ggcctccaca
 601  gccagtgacg tctacagctt cgggatccta atgtgggcag tgcttgctgg aagagaagtt
 661 gagttgccaa ccgaaccatc actcgtgtac gaagcagtgt gcaacaggca gaaccggcct
 721 tcattggctg agctgcccca agccgggcct gagactcccg gcttagaagg actgaaggag
 781 ctaatgcagc tctgctggag cagtgagccc aaggacagac cctccttcca ggaatgccta
 841 ccaaaaactg atgaagtctt ccagatggtg gagaacaata tgaatgctgc tgtctccacg
 901 gtaaaggatt tcctgtctca gctcaagagc agcaatagga gattttctat cccagagtca
 961 ggccaaggag ggacagaaat ggatggcttt aggagaacca tagaaaacca gcactctcgt
1021 aatgatgtca tggtttctga gtggctaaac aaactgaatc tagaggagcc tcccagctct
1081 gttcctaaaa aatgcccgag ccttaccaag aggagcaggg cacaagagga gcaggttcca
1141 caagcctgga cagcaggcac atcttcagat tcgatggccc aacctcccca gactccagag
1201 acctcaactt tcagaaacca gatgcccagc cctacctcaa ctggaacacc aagtcctgga
1261 ccccgaggga atcagggggc tgagagacaa ggcatgaact ggtcctgcag gaccccggag
1321 ccaaatccag taacagggcg accgctcgtt aacatataca actgctctgg ggtgcaagtt
1381 ggagacaaca actacttgac tatgcaacag acaactgcct tgcccacatg gggcttggca
1441 ccttcgggca aggggagggg cttgcagcac cccccaccag taggttcgca agaaggccct
1501 aaagatcctg aagcctggag caggccacag ggttggtata atcatagcgg gaaataa
```

FIGURE 5

```
  1 MSCVKLWPSGAPAPLVSIEELENQELVGKDGFGTVFRAQHRKWGYDVAVKIVNSKAISRE
 61 VKAMASLDNEFVLRLEGVIEKVNWDQDPKPALVTKFMENGSLSGLLQSQCPRPWPLLCRL
121 LKEVVLGMFYLHDQNPVLLHRDLKPSNVLPDPELHVKLADFGLSTFQGGSQSGTGSGEPG
181 GTLGYLAPELFVNVNRKASTASDVYSFGILMWAVLAGREVELPTEPSLVYEAVCNRQNRP
241 SLAELPQAGPETPGLEGLKELMQLCWSSEPKDRPSFQECLPKTDEVFQMVENNMNAAVST
301 VKDFLSQLKSSNRRFSIPESGQGGTEMDGFRRTIENQHSRNDVMVSEWLNKLNLEEPPSS
361 VPKKCPSLTKRSRAQEEQVPQAWTAGTSSDSMAQPPQTPETSTFRNQMPSPTSTGTPSPG
421 PRGNQGAERQGMNWSCRTPEPNPVTGRPLVNIYNCSGVQVGDNNYLTMQQTTALPTWGLA
481 PSGKGRGLQHPPPVGSQEGPKDPEAWSRPQGWYNHSGKZ
```

FIGURE 6

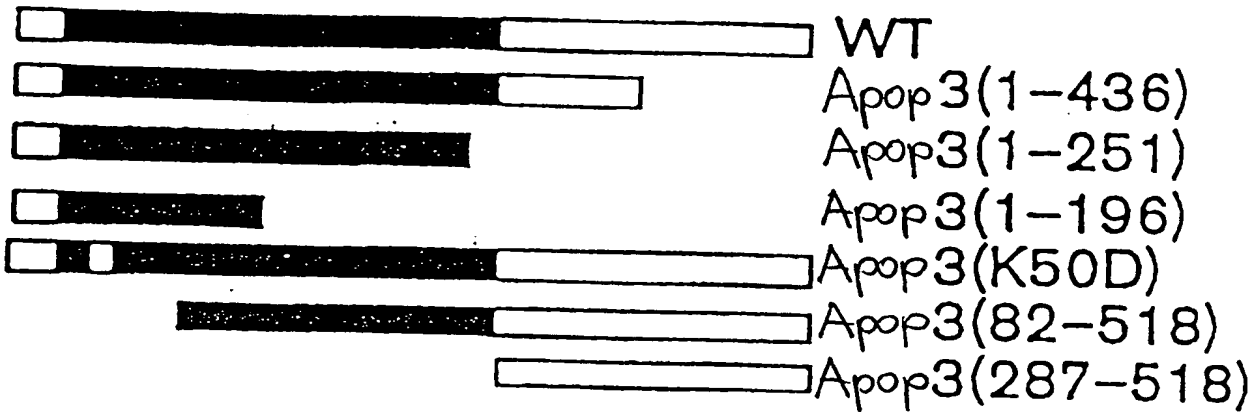
WT
Apop3(1-436)
Apop3(1-251)
Apop3(1-196)
Apop3(K50D)
Apop3(82-518)
Apop3(287-518)

Figure 7

	Binding to RIP
Apop 3	++++
Apop3(1-436)	+
Apop 3(1-251)	-
Apop 3(1-196)	-
Apop3(K50D)	++++
Apop 3(82-518)	+++
Apop3(287-518)	-

Figure 8

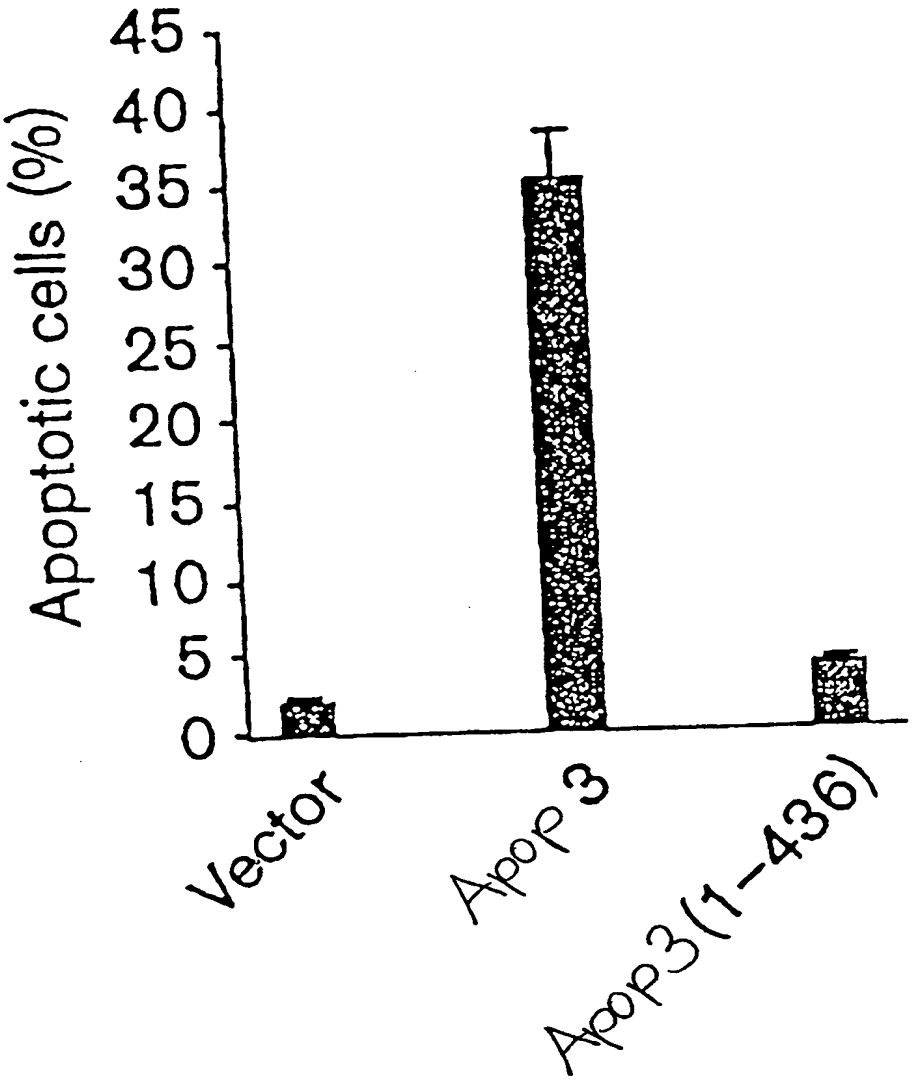
Apoptotic cells (%)
45
40
35
30
25
20
15
10
5
0
Vector
Apop 3
Apop3(1-436)

Figure 9

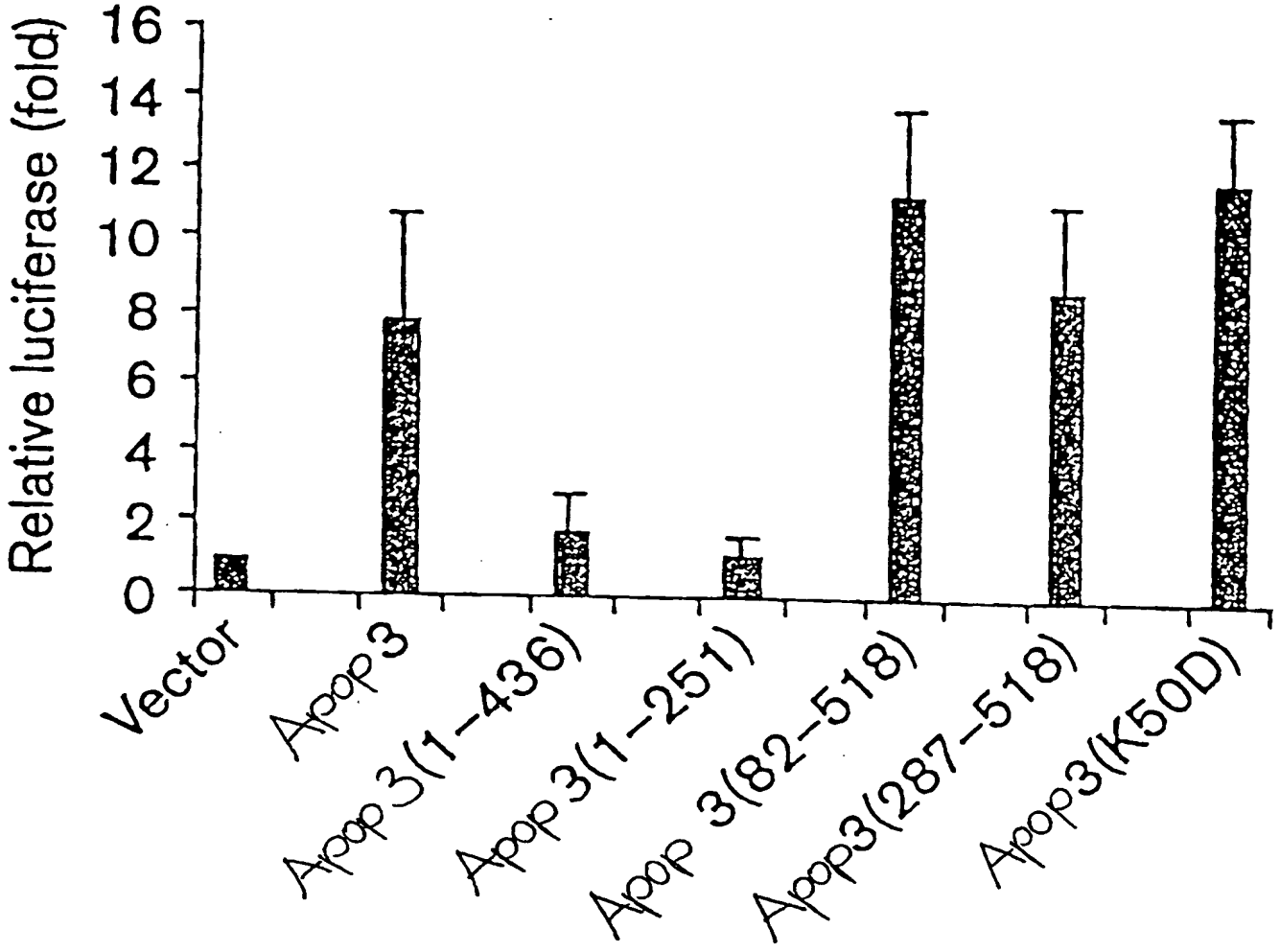
Relative luciferase (fold)
0
2
4
6
8
10
12
14
16
Vector
Apop3
Apop3(1-436)
Apop3(1-251)
Apop 3(82-518)
Apop3(287-518)
Apop3(K50D)

Figure 10

CORRECTED VERSION*

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(51) International Patent Classification 7 :
C07K 14/47, C12N 15/12, 5/10, C07K 16/18, G01N 33/50

A3

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(30) Priority Data:

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(71) Applicant *(for all designated States except US)*: RIGEL PHARMACEUTICALS, INC. [US/US]; 240 East Grand Avenue, South San Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants*(for US only)* : LUO, Ying [CN/US]; 32 Chester Circle, Los Altos, CA 94022 (US). HUANG, Betty, C., B. [US/US]; 771 Douglas Street, San Leandro, CA 94577 (US). SHEN, Mary [US/US]; 5670 Geranium Court, Newark, CA 94560 (US). YU, Pei, Wen [US/US]; 808 Comet Drive, #15, Foster City, CA 94404 (US).

(74) Agents: SILVA, Robin, M. et al.; Flehr Hohbach Test Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: APOPTOSIS PROTEINS

(57) Abstract

The present invention is directed to novel apoptosis polypeptides such as the Apop1, Apop2, and Apop3 proteins and related molecules which are involved in modulating apoptosis and to nucleic acid molecules encoding those polypeptides. Also provided herein are vectors and host cells comprising those nucleic acid sequences, chimeric polypeptide molecules comprising the polypeptides of the present invention fused to heterologous polypeptide sequences, antibodies which bind to the polypeptides of the present invention and to methods for producing the polypeptides of the present invention. Further provided by the present invention are methods for identifying novel compositions which modulate the biological activity of Apop1, Apop2, and Apop3, and the use of such compositions in diagnosis and treatment of disease.

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INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 99/17776

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/12 C12N5/10 C07K16/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, SCISEARCH, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "Human cDNA clone IMAGE:667313 5'" EMBL SEQUENCE DATABASE, 27 February 1997 (1997-02-27), XP002141903 HEIDELBERG DE Accession Nr.: AA227673; 99,3% identity seq.id.no.5, nucl. 940-1380 abstract --- -/--	2,5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2000	20/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/17776

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL.: "Human cDNA clone IMAGE:310581, 3'" EMBL SEQUENCE DATABASE, 19 April 1996 (1996-04-19), XP002141904 HEIDELBERG DE Accession No.: N99896 94,5% identity seq.id.no.5, nucl. 1289-1557 abstract	2,5
A	WO 96 36730 A (GEN HOSPITAL CORP) 21 November 1996 (1996-11-21) the whole document, especially page 13, lines 14-20 and page 20, line 17 - page 21, line 9	1,18
A	WO 97 15586 A (TULARIK INC) 1 May 1997 (1997-05-01) the whole document	1,18
A	COHEN G M: "Caspases: the executioners of apoptosis" BIOCHEMICAL JOURNAL,GB,PORTLAND PRESS, LONDON, vol. 326, 1997, pages 1-16, XP002107845 ISSN: 0264-6021 abstract; figure 4	1,17-19
P,X	STRAUSBERG R ET AL.: "Human cDNA clone IMAGE:2108297, 3'" EMBL SEQUENCE DATABASE, 5 February 1999 (1999-02-05), XP002141905 HEIDELBERG DE Accession No.: AI394293 100% identity seq.id.no.5, nucl. 1240-1557 abstract	2,5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr...onal Application No
PCT/US 99/17776

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636730 A	21-11-1996	US 5674734 A	07-10-1997
		AU 707598 B	15-07-1999
		AU 5487396 A	29-11-1996
		CA 2219984 A	21-11-1996
		EP 0852627 A	15-07-1998
		JP 11506317 T	08-06-1999
WO 9715586 A	01-05-1997	AU 7457796 A	15-05-1997

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(30) Priority Data:

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(74) Agents: SILVA, Robin, M. et al.; Flehr Hohbach Test Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: NOVEL APOPTOSIS PROTEINS

(57) Abstract

The present invention is directed to novel apoptosis polypeptides such as the Apop1, Apop2, and Apop3 proteins and related molecules which are involved in modulating apoptosis and to nucleic acid molecules encoding those polypeptides. Also provided herein are vectors and host cells comprising those nucleic acid sequences, chimeric polypeptide molecules comprising the polypeptides of the present invention fused to heterologous polypeptide sequences, antibodies which bind to the polypeptides of the present invention and to methods for producing the polypeptides of the present invention. Further provided by the present invention are methods for identifying novel compositions which modulate the biological activity of Apop1, Apop2, and Apop3, and the use of such compositions in diagnosis and treatment of disease.

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CLAIMS

We claim:

1. A recombinant nucleic acid encoding an Apop3 protein that is at least about 85% identical to the amino acid sequence depicted in Figure 6.

2. A recombinant nucleic acid that is at least about 85% identical to the nucleic acid sequence depicted in Figure 5 or its complement.

3. A recombinant nucleic acid according to claim 1, wherein said Apop3 protein is a human Apop3 protein.

4. A recombinant nucleic acid according to claim 1 encoding the amino acid sequence depicted in Figure 6.

5. A recombinant nucleic acid according to claim 2 that will hybridize under high stringency conditions to the nucleic acid sequence depicted in Figure 5 or its complement.

6. A recombinant nucleic acid according to claim 1 operably linked to control sequences recognized by a host cell transformed with the nucleic acid.

7. An expression vector comprising the nucleic acid of any claims 1-6.

8. A host cell comprising the nucleic acid of any claims 1-7.

9. A process for producing an Apop3 protein comprising culturing the host cell of claim 8 under conditions suitable for expression of an Apop3 protein.

10. A process according to claim 9, further comprising recovering said Apop3 protein.

11. A recombinant Apop3 protein that is at least about 85% identical to the amino acid sequence depicted in Figure 6.

12. An Apop3 protein according to claim 11 comprising the sequence depicted in Figure 6.

13. An Apop3 protein according to claim 11 or 12 encoded by a nucleic acid that is at least about 85% identical to the nucleic acid sequence depicted in Figure 5.

14. An Apop3 protein according to claim 11, 12 or 13 encoded by a nucleic acid that will hybridize under high stringency conditions to the complement of the nucleic acid sequence depicted in Figure 5.

15. A monoclonal antibody which specifically binds to an Apop3 protein according to claim 11, 12, 13, or 14.

16. A monoclonal antibody according to claim 15 that reduces or eliminates the biological function of said Apop3 protein.

17. A method for screening for a bioactive agent capable of binding to an Apop3 protein, said method comprising:

a) combining an Apop3 protein and a candidate bioactive agent; and

b) determining the binding of said candidate bioactive agent to said Apop3 protein.

18. A method for screening for a bioactive agent capable of interfering with the binding of Apop3 and RIP, said method comprising:

a) combining an Apop3 protein, a candidate bioactive agent and a RIP protein; and

b) determining the binding of said Apop3 protein and said RIP protein.

19. A method for screening for a bioactive agent capable of modulating the activity of an Apop3 protein, said method comprising the steps of:

a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding an Apop3 protein; and

b) determining the effect of the candidate bioactive agent on apoptosis.

20. A method according to claim 19, wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding an Apop3 protein.

09/762491

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
FP-68285/RMS		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/17776	06/08/1999	06/08/1998
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant RIGEL PHARMACEUTICALS, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06/03/2000	10.11.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Renggli-Zulliger, N Telephone No. +49 89 2399 7482

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/17776

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-50	as originally filed				

Claims, No.:

1-26	as received on	11/10/2000	with letter of	11/10/2000	

Drawings, sheets:

1/8-8/8	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-26
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	1-26
	No:	Claims	-
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	-

2. Citations and explanations
   see separate sheet

Re Item II

Priority

3. Additional observations :

The earliest priority document (6 August 1998) pertaining to the present application only discloses Sequence ID n°1 and n°2 (as filed) corresponding to Apop1.

The second priority document (8 September 1998) discloses the protein sequence of Apop3 (Figure 2) that corresponds to Seq. ID n°6 or Figure 6 of the present application as filed, except that the last amino acid of the sequence (glx) is not disclosed in the priority document. With respect to the nucleotide sequence, the priority document (8 September 1998) discloses a polynucleotide sequence (Figure 1) that does not correspond to the Seq. ID n°5 or Figure 5 of the present application as filed.

Therefore, both priorities are not valid for the subject-matter of claims 1-20 referring to the recombinant nucleic acid and protein sequences of Apop3.

The P/X documents D3 (5 February 1999) (see Re Item V of this written opinion for the numbering of the cited documents) published before the filing date (6 August 1999) of the present application is considered to be part of the prior art in the sense of Rule 64.1(a) and (b)(i)(ii) PCT and relevant to assess novelty and inventive step according to Article 33 (2) and (3) PCT.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: HILLIER L ET AL.: 'Human cDNA clone IMAGE:667313 5" EMBL SEQUENCE DATABASE, 27 February 1997.

D2: HILLIER L ET AL.: 'Human cDNA clone IMAGE:310581, 3" EMBL SEQUENCE DATABASE, 19 April 1996 .

D3: STRAUSBERG R ET AL.: 'Human cDNA clone IMAGE:2108297, 3" EMBL SEQUENCE DATABASE, 5 February 1999.

Novelty (Article 33(1) and (2) PCT) and Inventive step (Article 33(1) and (3) PCT)

1) D1 discloses a nucleotide sequence (N99896) of unknown function that is 94.5% identical to Seq. ID n°5/Figure 5 (1557bp) in a 271bp overlap.
D2 discloses a nucleotide sequence (AA227673) that is 99.3% identical to Seq. ID n°5 (1557bp) in a 441bp overlap.
D3 discloses a nucleotide sequence (AI394293) that is 100% identical to Seq. ID n°5 (1557bp) in a 312bp overlap.

These EST sequences (D1-D3), that are considered as the closest prior art, are of unknown function and do not encode an Apop3 protein of amino acid sequence as depicted in Figure 6. Furthermore, the remaining prior art does not mention the possible existence of this novel protein (Apop3). Therefore, the subject-matter of claims 1-26 is considered novel and inventive.